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THE ACTION OF PROTEOLYTIC ENZYMES ON WHEAT GLUTEN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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by
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ABSTRACT

Sedimentation characteristics of gluten dispersed in an acidic medium, neutral solvents, and an alkaline medium were found to be similar.

Sedimentation analysis was used to follow the proteolysis of gluten dispersed in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml., and in 0.02 M aluminum lactate buffer, pH 3.05. Papain and a neutral protease present in Fermex MT were found to act in the former solvent whereas pepsin and the acidic protease of Fermex MT acted in the latter.

The sedimentation coefficient of the enzymic digests decreased as the reaction time increased, indicating a progressive decrease in molecular size. The amount of protein digested showed a progressive increase with time.

Action of the neutral protease of Fermex MT was different from that of the acid protease. There was a greater variation in the molecular size of the products of hydrolysis produced by the neutral protease. Action of papain was found to be similar to that of the neutral protease of Fermex MT.

The action of pepsin was different from that of the acid protease of Fermex MT. Pepsin hydrolysed gluten to a greater extent than did the acid protease of Fermex MT, but the products of hydrolysis did not show as much variation in the molecular size as those obtained with the acid protease.

In an experiment with four gluten fractions (obtained by successive additions of saturated magnesium sulphate solution to gluten dispersion in sodium salicylate containing sodium bisulfite), the action of the neutral protease of Fermex MT on the more insoluble fraction was found to be different from the action on the more soluble fractions.

An enzyme resistant 'core', insoluble in conventional solvents and low in amide-nitrogen content, was found.

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INTRODUCTION

Irvine (1) at the Grain Research Laboratory, Winnipeg, observed an improvement in the quality of bread baked from aged flour, when fungal enzymes were added to the dough. These observations were confirmed in this laboratory. A maximum increase of 165 c.c. in loaf volume was obtained on a 100 gm. loaf to which fungal enzymes had been added. The increase in volume was accompanied by an improvement in the quality of the enzyme-containing doughs and in the gluten obtained from such doughs. The addition of fungal enzymes obviously changed the characteristics of the gluten and it was decided to study the nature of these changes. As very little information regarding the action of proteolytic enzymes on wheat gluten is available in the literature, it was deemed necessary to study this action before making an attempt at explaining the above observations. The present investigations have, however, been confined to the effects of proteolytic enzymes on gluten obtained from normal wheat flour.

LITERATURE REVIEW

A. Effect of proteolytic enzymes on gluten

Hildebrand (2) has reviewed the role of proteases in baking. This article indicates that very little information is available on the action of proteolytic enzymes on wheat proteins although there have been many investigations designed to secure information on the presence of proteolytic enzymes in wheat, wheat flour and other sources of enzymes used in baking, and on the effect of proteases on quality of bread.

Balland (3) made the first attempt to study the effect of proteolytic enzymes on gluten. By mixing the dough with water extract of sprouted wheat flour and washing out the gluten after different lengths of time, he found that the amount of gluten obtained decreased as the time interval between doughing and washing out was increased. He, therefore, concluded that the sprouted wheat flour contained a protein-splitting enzyme. Baker and Hulton (4) showed that flours containing peptic enzymes produced poor quality bread. From the liquefaction of gelatin by flours possessing poor baking quality, Ford and Guthrie (5) concluded that the weakness of the flours was due to the proteoclastic enzymes which disintegrate gluten. Stockham (6) arrived at a similar conclusion by observing the time taken for liquefaction of 1.5% gelatin by different grades of flours. He

was of the opinion, however, that the differences between different classes of wheats were not due to their proteolytic activity.

Collatz (7) demonstrated that the addition of a malt preparation to wheat flours increases their proteolytic activity.

Sharp and Elmer (8) studied the proteolytic degradation of different proteins of wheat flour by the enzymes present in it. They determined the amounts of protein soluble in 5% potassium sulphate, in 70% alcohol, and insoluble in these two reagents, and the amounts of amino-nitrogen over a period of five weeks. They concluded that the proteolytic enzymes of the flour are capable of digesting the flour proteins if given sufficient time and that auto-digestion of the flour for a considerable period of time results in a decrease of gliadin, an increase in potassium-sulphate-soluble fraction and no change in glutenin. There was also an increase in amino-nitrogen. From these results it appeared that the albumins, globulins and gliadin of wheat flour were quite susceptible to the proteolytic enzymes of the flour whereas glutenin was not attacked.

Bailey and associates published the results of their studies over a period of years (9, 10, 11, 12, 13, 14). Olsen and Bailey (9) did not find any appreciable effect of

the proteases contributed by normal intact yeast cells on gluten proteins during a period of 4-5 hours fermentation. Cairns and Bailey (10) demonstrated that out of eight chemical methods used for following the proteolysis in flour, Sorensen's formol titration method was the best. The increase in amino-nitrogen, hence the proteolysis, in high grade flour suspensions over a period of 48 hours at 37° C. was small, but sprouted wheat flour showed very high proteolytic activity, and the addition of sprouted wheat flour to high grade flour suspension resulted in an increased rate of proteolysis. They also found a positive correlation between the ash content and proteolytic activity of the flour. Brownlee and Bailey (11) did not find any change in the peptization of dough proteins with various salt solutions on the addition of small amounts of proteases. Stannous chloride precipitation of true proteins did not show any change in the gluten proteins during ordinary fermentation of the dough, but a slight cleavage of the gluten proteins occured in doughs to which Diamalt had been added. The use of copper sulphate, which precipitates more protein than does stannous chloride, showed substantial proteolysis in doughs to which pepsin had been added. Tissue and Bailey (12) confirmed Cairns and Bailey's (10) observation that formol titration is a suitable method for following the rate of proteolysis in flour suspensions. They found that the diastatic malt preparations had high proteolytic

activity and that, if used in sufficient quantities to improve the diastatic activity of flour, produced an inferior grade of bread, apparently because of proteolytic degradation of proteins. Addition of small amounts of papain to the doughs did not affect their stress readings, although a considerable decrease was found with larger additions (13). The elasticity and the stress readings of the doughs were decreased on the addition of barley malt extract and malted wheat flour. These additions also increased the mobility of the dough. Rupp and Bailey (14) found a proportional decrease in the development work on the addition of increasing amounts of papain to the doughs. They also found that the amount of pepsin required to produce an effect equal to that produced by a constant amount of papain, varied from flour to flour, and concluded that the action of pepsin was different from that of papain.

Read and Haas (15, 16, 17, 18) observed definite improvements in 'bucky' doughs on the addition of proteolytic enzymes and yeast autolysates. They showed that excessive additions of proteinases or proteinase activators ruin the baking properties of flours and that the activity of the proteases such as papain, bromelin and others could be repressed by the addition of comparatively large amounts of oxidising agents, especially potassium bromate.

Balls and Hale (19) and Jørgensen (20) demonstrated the presence of proteolytic enzymes in wheat flour and found that these could be activated by reducing agents such as glutathione and cysteine. Balls and Hale (19) also showed that both papain and glutathione liquified dough when added to it and the loaf did not rise at all. They also showed that the activity of the proteolytic enzymes of flour could be reduced by the addition of oxidising agents.

Blagoveschensky and Sossiedov (21) demonstrated the presence of a protein disaggregating enzyme in the flour obtained from wheat damaged by "tortoise bug" (Eurygaster species). Gluten obtained from such flour disaggregated rapidly without any noticeable increase in amino-nitrogen. Blagoveschensky and Yurgenson (22) found that during fermentation of the dough there is a marked increase in water-soluble nitrogen and a decrease in the gliadin nitrogen. They determined the effect of flour enzymes on the different proteins of dough at pH values of 3.8, 4.8, and 5.1, and found that the water-soluble nitrogen increased at the expense of other protein fractions, the decrease being most marked in gliadin and least in the case of glutenin. However, they considered that these changes in the protein solubility were not a result of the proteolysis by the flour enzymes but rather of a protein disaggregating action. The proteolytic enzymes of yeast did not have any action on the

different protein fractions at a pH of 8.5, though they showed proteolytic activity between the pH range of 4.8 - 6.5. Later, these workers presented the results of the action of flour and yeast proteinases on isolated leucosin, gliadin and glutenin (23). They found that the action of these enzymes on different flour proteins was a disaggregating one rather than true proteolysis, as evidenced by a very insignificant increase in amino-nitrogen. The optimum pH for the action of proteases differed for each protein.

Flohil (24) prevented the deterioration in the baking quality of a flour containing 0.005% papain, by the addition of potassium bromate. He found that both papain and water extract of malted wheat flour completely broke down gluten within 48 hours at 30° C. He demonstrated that the proteolytic activity of barley malt sprouts was greater than that of the wheat germ, which had an effect similar to that produced by papain, if it were included in a dough.

Harris (25) determined the rate of solubility of gluten, in 10% sodium salicylate obtained when different concentrations of papain, yeast extract, pepsin, pancreatin, malt diastase, takadiastase and bromelin were mixed in a dough and the gluten washed out immediately with 0.1% phosphate buffer solution at a pH of 6.8 - 7.0. He found that in most instances the inclusion of enzymes hastened the rate of dispersal of gluten in sodium salicylate

solution and that this rate was further accelerated as the concentration of the enzymes was increased. Bromelin and pancreatin had the greatest influence on the solubility of the gluten while takadiastase appeared to decrease it. This reduction in solubility of gluten was explained as being due to the coagulation of the proteins by takadiastase. Potassium bromate had inhibitory effects on papain and bromelin, although when used in low concentrations it seemed to activate pepsin and some other enzymes. Potassium bromate did not inhibit the activity of papain at higher concentrations. Harris and Johnson (26) found that incorporation of papain and pepsin in doughs increases the viscosity of gluten dispersed in 10% sodium salicylate. They found that the amount of protein precipitated by addition of a constant volume of concentrated magnesium sulphate solution decreased with increasing amounts of papain, but did not change appreciably with increased concentrations of pepsin. This they believed was due to the differences in the action of the two enzymes. Addition of potassium bromate to enzyme-containing doughs altered the rate of dispersion and lowered the viscosity of dispersions. Later on they reported (27) that the rate of dispersion of gluten was slower after three-hour fermentation. This effect could be obliterated by adding papain. Addition of papain lowered the resistance of the doughs while the addition of potassium bromate increased it.

From farinograph studies with doughs containing glutathione or papain, Ford and Maiden (28) concluded that the action of these differs. The immediate softening action of glutathione did not increase with time, and these workers concluded its action was on the proteins and not on the proteases of flour. The effect of papain was immediate, and, in addition the doughs to which papain had been added showed progressive increase in softening with time.

Freilich and Frey (29) showed that the proteolytic activity of flour proteinases and of added papain could be retarded by mixing the dough under oxygen, even though oxygen did not have any effect on papain as was shown by mixing papain exposed to oxygen outside the dough. They used formol titration for determining the extent of proteolysis. They also demonstrated that the addition of potassium bromate could overcome the deterioration in the baking quality of doughs caused by added papain.

Elion (30) confirmed Ford and Maiden's (28) observation that the softening effect of papain was immediate and stronger than that of glutathione, if both were incorporated in the dough. But, in contrast to their opinion, he believed that glutathione acted as an activator of proteolytic enzymes, as no softening occured if glutathione was added to a heated flour. Heat treatment, according to him, destroyed the proteolytic enzymes of flour.

Olcott, Saperstein and Blish (31) determined the viscosity of gluten dispersed in dilute acetic acid and sodium salicylate solution, before and after heating the dispersions. They observed a decrease in the viscosity of the unheated acetic acid dispersions with time. There was no decrease in the viscosity of heated acetic acid dispersion. They determined the non-protein nitrogen and amino-nitrogen content of the acetic acid dispersions and found a progressive increase in both the values in the case of unheated dispersions. The decrease in the viscosity of the unheated acetic acid dispersions and increase in the non-protein and amino-nitrogen content of such dispersions was ascribed to a proteolytic enzyme which was inactivated on heating. The viscosity of sodium salicylate dispersion did not change, but when such a dispersion was dialysed against acetic acid solution, it was found that the viscosity of the dispersion so obtained decreased. These workers concluded that sodium salicylate reversibly inactivates the proteolytic enzyme of gluten.

Sandstedt and associates (32, 33, 34, 35) have investigated the relation between proteolysis and oxidation and reduction of doughs. Sandstedt and Fortmann (32) concluded that the degradation of gluten proteins in doughs brought about by added papain was different from that of reducing agents as the former could not be reversed by

subsequent oxidation. Hites, Sandstedt and Schaumburg (33) found that proteolysis in bread doughs, caused by papain, started only after 6 mg. percent of papain had been added. They also showed that flour contained a water-soluble papain inhibitor, which occurred in greatest concentrations in wheat bran. Later (34) these workers showed that the flour proteinases which had been considered to be papainases (19) were actually different because they did not respond to the action of reducing and oxidizing substances as did added papain. Sandstedt and Mattern (35) conducted baking tests and confirmed the findings of Sandstedt and Fortmann (32) that the action of added papain could not be reversed by subsequent oxidation whereas that of the reducing agents could be reversed, though both produced similar visual effects.

Swanson and Andrews (36) studied the action of papain, yeast water, cysteine and glutathione on flour. They found that the mixogram patterns produced by all these were similar with continuous mixing but the patterns obtained after rest periods showed distinct differences where papain was added. They also determined the amount of gluten which could be recovered from the doughs treated with various substances and found that the amount of gluten recovered from doughs treated with papain decreased as the amount of papain was increased or the rest period was lengthened.

Such effects were not observed with yeast water, cysteine or glutathione. The reduction in the amount of gluten recovered from papain-treated doughs was attributed to the proteolytic disintegration of gluten.

Stern (37) confirmed the findings of Ford and Maiden (8), and Swanson and Andrews (36) that the breakdown of gluten does not proceed beyond a certain point by the addition of reducing agents whereas increasing amounts of papain cause progressive breakdown of gluten, as shown by increase in soluble nitrogen and decrease in nitrogen obtained as gluten.

Miller and associates have presented the results of their studies of the action of proteolytic enzymes from various sources on wheat flour proteins in a number of papers (38, 39, 40, 41, 42, 43, 44, 45, 46, 47). Miller and Johnson (38) found that the action of proteolytic enzymes is different in sponge doughs from that in straight doughs. High concentrations of alpha-amylase extracts containing large amounts of proteolytic enzymes when added to sponge doughs, reduced the remix time, made the dough extremely soft and sticky, and resulted in a very inferior loaf. Similar amounts did not cause any harmful effects on the quality of bread produced by straight dough procedure. They were able to counteract the effects of proteolytic enzymes in sponge doughs by the addition of sodium chloride and

found that approximately 55% of the proteolytic activity could be inhibited by the addition of salt. Potassium bromate was not found to affect the proteolytic activity of the enzymic supplements. Malted wheat flour and a fungal preparation were used as sources of alpha-amylase. They (39) inactivated selectively the amylolytic and proteolytic enzymes of malted wheat flour, malted barley and a fungal concentrate. Inactivation of proteolytic enzymes was accomplished by raising the pH of the extracts to 9.3 - 10.2, and heating the extracts to 50° - 60° C. In some preparations the proteinases were inactivated if the temperature of the alkaline extracts was lowered to 5° C. Using the above-mentioned method of inactivation of proteinases, they demonstrated (40) that the decrease in dough consistency was mainly due to the alpha-amlylases, at normal levels of malt supplements. They observed a decrease in dough consistency with increased amounts of proteases. The effects of increasing the concentration of protease differed with the malted wheat flour and the fungal extracts and with the flour employed. A marked increase in the loaf volume occurred with hard red spring wheat flour where as there was no change in winter wheat. In later publications (41, 42, 43, 44) these authors confirmed their earlier results and advanced an explanation for the increased extensibility of doughs after the action of proteolytic

enzymes. They suggest (44) that the proteolytic enzymes break the peptide linkages in the amino acid chain resulting in increased extensibility.

Abbott, Miller and Johnson (45) used a spectrophotometer to determine the extent of proteolysis of wheat gluten by papain, malted wheat flour and a fungal concentrate. They found an increase in the optical density (at 270-275 mu) of the filtrates obtained from proteolytic digestion of gluten, with increasing amounts of the enzymes. They showed that different enzymes acting on gluten produce soluble chromophoric material at different rates from the release of soluble nitrogen, indicating that the sites of action of the enzymes are different. Johnson and Miller (46) used the farinograph to follow the action of papain, malted wheat flour, pepsin and some commercial fungal enzyme preparations, having different proteolytic activity. They demonstrated that α-amylase free, fungal preparation Rhozyme P-11 had optimum proteolytic activity in sponge doughs at a pH of 4.12. At a pH of 4.7 the same protease decreased the consistency of the dough as the temperature was increased from 25° C. to 40° C. The consistency of the sponge doughs, after a four-hour digestion at pH 4.7 and 30° C., decreased as the concentration of the protease was increased. Similar effects on the dough consistency were obtained by increasing concentrations of pepsin, malted wheat flour proteinase and Rhozyme-S. They did

not, however, obtain a linear relationship between the decrease in dough consistency and increase in the amounts of papain until a comparatively higher dose of papain had been reached. In their opinion this effect confirms the presence of an inhibitor in the flour (33) which, however, does not have any effect on the activity of pepsin, fungal, or malted wheat proteinase. Potassium iodate had an inhibitory effect on papain, as shown both by determination of dough consistency and Ayre-Anderson (48) procedure using gluten or hemoglobin substrates. No inhibitory effect of potassium iodate was observed in the case of fungal proteinases. Malted wheat flour was shown to contain two proteinases, one of which could be inactivated by potassium iodate or potassium bromate. This was shown by the Ayre-Anderson (48) method using gluten as the substrate. Johnson, Miller, Boyer and Geddes (47) have reported the action of various proteases on wheat gluten, in acetic acid - acetate buffer and disodium phosphate - monopotassium dihydrogen phosphate buffer. Dried undenatured gluten was hydrolysed by fungal and malted wheat flour proteases to a lesser extent than was hemoglobin. Calculation of the reaction rate constants suggested that this was due to the insolubility of gluten in the solvents used. Denaturation of gluten by heating the wet crude gluten at 150° C. for 4 hours reduced the solubility in acetate buffer by 60% and further reduced the

hydrolysis by malted wheat flour protease. An increase in the amount of gluten as substrate resulted in inhibition of gluten hydrolysis by malted wheat or barley flour proteinase. This reduction of proteolysis was not observed with Rhozyme-S, pepsin, unactivated papain, colase or even malted wheat and barley flour extracts freed from alpha-amylase. This seemingly confirmed the presence of an inhibitor previously reported (33, 46). A calculation of Michaelis-Menten constants showed that the enzymes, Rhozyme-S and malted wheat flour proteinase had greater affinity for gluten than for hemoglobin and the maximum velocity of reaction was greater for hemoglobin than for gluten. This explains why gluten is hydrolysed less rapidly than hemoglobin. The apparent activation energy values calculated for the action of various proteases on gluten were found to be very similar indicating that these enzymes hydrolyse similar bonds in gluten. These workers also reported optimum pH values for the action of various proteolytic enzymes on gluten. For malted wheat flour and fungal protease the pH optimum was between pH 3.0 and 4.0, for trypsin it was near pH 7.0 - 8.0, and for papain it was around pH 5.5.

In a recent publication Strusi (49) has reported the results of tryptic hydrolysis of gluten obtained from durum and bread wheats. After 24 hours of enzyme action on the two types of gluten, he found more solid residue in the case of durum wheat than in the case of bread wheat,

and when the reaction time was extended to 96 hours, the enzymic hydrolysate from soft wheat was milky and that from durum wheat was clear. On circular paper chromatography of the enzymic hydrolysates, he observed that there were differences between the two. In one set of the chromatograms there were eight bands from each type of gluten. In the case of durum wheat there were five bands which he attributed to peptones while in the case of vulgare wheat there were only four. The other bands were due to amino acids. Bands 6, 7, and 8 in the case of durum wheat were tyrosine, valine and leucine respectively, while bands 5 - 8 from vulgare wheat were tyrosine, valine, phenylalanine and leucine. Similarly, in the other pair of chromatograms there were five peptones in the case of durum wheat gluten whereas there were only four for the tryptic hydrolysate of bread wheat gluten. Bands 6, 7, 8, 9, and 10 were respectively alanine, tyrosine, valine, phenylalanine, and leucine in the enzymic hydrolysate of durum wheat gluten. The same amino acids were found in the same order in bands 5 - 9 of the chromatogram for bread-wheat gluten. On the basis of these observations the author suggests that the differences in the gluten from the two types of wheat may lie in their molecular configuration.

B. Fractionation of gluten

Many workers using different techniques, have fractionated wheat gluten in attempts to characterise it. Earlier work on fractionation of wheat gluten has been reviewed by Osborne (50) and Bailey (51). Even though most of it is only of historical interest it seems desirable to include a brief summary. Taddei (50) reported the presence of an alcohol-soluble protein in the gluten, and named it 'gliadin'. To the alcohol-insoluble protein on the wheat gluten he gave the name 'zymom'. Ritthausen (50) considered gluten to be composed of four different proteins, gluten-casein, gluten-fibirin, plant-gelatin or gliadin and mucedin. Gluten-casein was insoluble in water, very slightly soluble in dilute alcohol, but dissolved readily in very dilute acids and alkali. Gluten-fibrin was that part of the alcohol soluble protein which was soluble in strongest alcohol, and in dilute acids and alkali. Gliadin, according to him, was that portion of the alcohol-soluble protein which dissolved freely in 60 - 70% alcohol, besides being soluble in dilute acids and alkali. Mucedin, the fourth protein occuring in wheat gluten, differed from plant-gelatin only in being more soluble in water and dilute alcohol. Osborne (50), himself, reported that gluten consisted of two well-defined proteins, gliadin and glutenin. He found gliadin to be insoluble in water, and neutral salt

solutions, but soluble in dilute alcohol, dilute acids and alkali. Glutenin on the other hand was insoluble in alcohol but soluble in dilute acids or alkali.

Results at variance with Osborne's (50) conception of two well-defined proteins, were obtained by Haugaard and Johnson (52) and by Harris and Bailey (53) who used thermal fractionation technique. Additional evidence for the view that gluten did not consist of only two well-defined proteins has been presented by many authors using various techniques. Only the results which were obtained in fractionation studies will be considered here.

Sandstedt and Blish (54) believed that gluten proteins are made up of three groups of protein, proteins of the gliadin group, of the mesonin group, and those of the glutenin group. This classification was based on 'certain predominating tendencies', even though sharp and definite boundaries were hard to obtain.

Fractional precipitation and fractional solubility have been used by McCalla and coworkers (55, 56, 57). McCalla and Rose (55) obtained five fractions from gluten dispersions in sodium salicylate by the addition of saturated magnesium sulphate solution to the dispersion. The smooth curve obtained for the amount of protein precipitated by increasing concentrations of magnesium sulphate suggested that the gluten did not consist of well-defined proteins. The amide- and arginine-nitrogen content of the different

fractions led them to believe that gluten consisted of a large number of components which differed systematically and progressively from each other. This statement, however, does not apply to the 10-15% most soluble protein present in the dispersions, which is low in amide nitrogen and high in arginine. Similar results were obtained by Spencer and McCalla (56) who used fractional solubility of the wheat gluten as the criterion. Results supporting this view were obtained by McCalla and Gralén (57) who used ultracentrifugation and diffusion for the characterisation of the gluten fractions precipitated from sodium salicylate dispersions by the method of McCalla and Rose (55).

Harris has published a number of papers on the fractionation of gluten. The first two of his papers (58, 59) used the technique of fractional precipitation as outlined by McCalla and Rose (55). By addition of different volumes of concentrated magnesium sulphate solution to gluten dispersed in 10% sodium salicylate solutions, he obtained varying amounts of protein with different varieties and types of wheat. He did not, however, present any new information regarding the nature of the protein present in the fractions. Later studies (60, 61) on fractionation were also carried out with a view to obtaining information regarding the baking behaviour of the gluten fractions. In one experiment (60) the proteins were precipitated at pH

values of 5.0, 5.0 - 5.5, and over 5.5, while in the latter work (61) the pH levels employed were 5.1, 5.1 - 5.8, and over 5.8. The slight increase in the alkalinity of the dispersion resulted in different amounts of protein being precipitated, indicating that the amount of protein precipitated as one fraction could be varied by slightly altering the conditions of precipitation. In a recent publication, Stauffer, Banasik, and Harris (62) have reported the results of the amino acid distribution in the fractions obtained by varying the pH levels of the gluten dispersion. Though significant variations in the content of certain amino acids were reported, it cannot be concluded from the results that the fractions represent distinct proteins.

Cunningham, Geddes, and Anderson (63) obtained smooth curves when they plotted the amount of protein precipitated from the formic acid extract of wheat flour against changes in salt concentration and variation in pH. These results support the contention (55) that gluten does not consist of a few well-defined proteins.

Jones, Taylor, and Senti (64) have published the results of fractionation studies employing variation in pH and ionic concentration, and extraction techniques. The electrophoretic examination of the fractions showed that all of them were heterogeneous. Recently Woychik, Dimler,

and Senti (65) have fractionated gluten into four fractions on carboxymethylcellulose columns. All the fractions were electrophoretically heterogeneous. Fractions II, III, and IV were rechromatographed, and the material so obtained showed electrophoretic homogeneity. This observation is the basis of their claim that the fractions represent 'true protein entities'. They, however, have not explained the occurrence of more than one peak in the elution patterns obtained on rechromatography of the fractions.

Meredith, Sammons, and Frazer (66, 67) using partial solubility in methanol-chloroform mixture and dilute formic acid, observed differences in the yield of fractions obtained from gluten prepared by two different methods. They, therefore, concluded that gluten is a complex which, when prepared according to the method of Cunningham _et al._ (68), dissociates into fractions.

Simmonds and Winzer (69) fractionated the acetic acid soluble portion of wheat gluten on a carboxymethylcellulose column. They obtained seven fractions. Two of the fractions β and ε accounted for about 85% of the total protein in the solution. The elution pattern of the β fraction, the only one to be rechromatographed, showed a single symmetrical peak. The authors, however, point out that the fraction may not be homogeneous when subjected to other methods of fractionation.

C. Solubilization of gluten proteins

Even after 200 years of research, the problem of solubility of wheat gluten has not been solved satisfactorily. Beccari's (70) announcement that gluten can be obtained by washing out the starch from a dough was followed by Einhof's (50) attempt to solubilize it in alcohol. He believed that the protein extracted by alcohol from wheat flour represented the gluten, but this belief proved to be partially wrong. Taddei (50) showed that gluten consists of an alcohol soluble protein and a protein insoluble in alcohol. Though Dumas and Cahours, and Ritthausen, amongst others, tried to solve this problem, none of them could add much to the existing knowledge. In 1907, Osborne published the results of his researches on proteins of the wheat kernel (50). He found that gluten contained an alcohol soluble protein and, in an equal amount, a protein which could be dissolved in dilute acids and alkalis, and could be precipitated by neutralization of such solutions. This classification on the basis of solubility was far from satisfactory as was shown by many later workers. Gortner, Hoffmann and Sinclair (70) found that by varying the concentration and nature of the salt solution, varying amounts of gluten proteins could be dissolved. Blish and Sandstedt (72, 73) showed that neither gliadin nor glutenin, as prepared by Osborne's (50) method, is an individual protein, and that the use of alkali in the preparation of glutenin

causes irreversible changes. Cook and colleagues published the results of their investigations in a number of papers (74, 75, 76, 77). They concluded that the gluten proteins, dispersed in neutral solvents such as urea and sodium salicylate solutions, retain the properties of native proteins to a higher degree than when dispersed in acid or alkaline solutions.

McCalla and associates reversibly fractionated gluten from sodium salicylate solutions (55, 56). They also found that a considerable portion of the gluten protein in sodium salicylate dispersion was in molecular dispersion and could be studied by physico-chemical means (57). Later (78), however, they showed that sodium salicylate is not a satisfactory dispersing agent if the electrophoretic properties of gluten are to be studied. Both Udy (79) and Olcott _et_ _al_. (31) showed that acetic acid dispersions of gluten were not stable and that gluten dispersed in sodium salicylate was stable. Udy (79), in addition, showed that sodium salicylate repressed the electrostatic charges on the gluten molecules, confirming Colvin and McCalla's (78) observation that gluten in sodium salicylate was electrophoretically homogeneous.

Vercourtern and Lontie (80) were able to dissolve gluten in 2, 4-dimethyl formamide, but the proteins precipitated out if the reagent was dialysed and the aqueous

solution allowed to stand at 0° C. Lontie, Creveour, and Dulcino (81) conducted electrophoretic studies with gluten dispersed in 0.015 M sodium lactate buffer, pH 3.5, containing 0.1% 2-mercaptoethanol.

Cunningham et al. (68) found that gluten could be extracted by dilute formic, citric and oxalic acid, but the protein precipitated on addition of salts (63).

Jones et al. (64) found that amongst many aluminum buffers used for electrophoretic study, 0.02 M aluminum lactate buffer at pH 3.1 was the most suitable for the study of gluten.

A number of workers (82, 83, 84) have reported a liquifying action of reducing agents on gluten. DeDeken and Mortier (85) were able to dissolve about 90% of the gluten in 0.01 M solution, pH 11, of thioglycollic acid, cysteine, and sodium sulphite. McDermott and Pace (86) were able to completely solubilize wheat gluten in Swan's reagent (87), cupric-ammonium-sulphite, and claimed that the proteins were in the form of water soluble derivatives.

MATERIALS AND METHODS

All experiments were carried out using ether-extracted flour from Red Bobs wheat, milled on an experimental Buhler mill. The protein content of the ether-extracted flour was 14.0% (N x 5.7).

Gluten was washed using a gluten-washing machine and 0.1% phosphate buffer at pH 6.8 as recommended by Dill and Alsberg (88). Dispersion of gluten was obtained by immersing the gluten ball in a known volume of dispersing agent (allowance being made for moisture in gluten), and stirring the solution occasionally. After all gluten had dispersed, the dispersion was kept overnight in a cold room at 3° C. Starch was removed by centrifuging at 9,500 g. for 20 minutes in an angle centrifuge at 3° C. The supernatant was kept at the same temperature until used.

Fisher certified chemicals were used in the preparation of the dispersing agents. Weighed amounts of sodium salicylate and sodium bisulfite were dissolved in water and made up to volume to give an 8% solution (w/v) of sodium salicylate containing 50 mg. sodium bisulfite per 100 ml. solution. Sodium salicylate solution (8%, w/v) without the bisulfite was prepared in the same manner. The pH of the solutions was between 6.6 - 6.8. Aluminum

lactate buffer was prepared according to the method of Jones et al. (64) by activating a known weight of aluminum foil in mercuric chloride solution and dissolving the activated aluminum in lactic acid. The solution so obtained was made up to volume so that the resulting concentration was 0.02 M in respect to aluminum. The pH was adjusted to 3.05 by the addition of lactic acid. Swan's reagent (87), cupric-ammonium-sulfite was prepared as follows:

1.9967 gm. of cupric acetate (monohydrate) containing 0.6357 gm. of copper was dissolved in distilled water and copper hydroxide precipitated by the addition of dilute ammonia. The precipitate was washed free of acetate with distilled water. The acetate-free precipitate of copper hydroxide was dissolved in a minimum volume of liquid ammonia and 3.1512 gm. of sodium sulfite added to the blue solution. The volume was made up to 500 ml., the resulting solution being 0.02 M in respect to copper and 0.05 M in respect of sulfite. The pH of the reagent was about 10.5.

Fermex MT, a commercial preparation of enzymes produced by Aspergillus oryzae contains two proteases. The pH optima for one protease is at pH 3.5 and for the other at pH 7.0. This preparation was obtained from Wallerstein Company*. Crystalline pepsin, 2x, was

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*The number and characteristics of the proteases in Fermex MT are based on a private communication from Wallerstein Company.

obtained from Worthington Biochemicals. Crude papain was obtained from Fisher Scientific Co.

Enzymic hydrolysis of gluten was carried out either in erlenmeyer flasks or in cellophane tubing. Weighed amounts of the enzymes were added to known volumes of gluten dispersion brought to the reaction temperature in a thermostatically controlled water-bath. Cellophane bags were used in dialysis experiments. The bag was tied to a rotatory dialyzer and immersed in a vessel containing the solvent brought to the required temperature in the water-bath. The amount of solvent used was 20-40 times that of the reaction mixture.

Fractionation of gluten dispersed in sodium salicylate, and in sodium salicylate containing sodium bisulfite was carried out essentially according to the procedure of McCalla and Rose (55), except that the fractions obtained were not washed before redispersion in 8% sodium salicylate. Calculated volumes of saturated magnesium sulphate solution were added to gluten dispersions to give 0.025, 0.05, 0.075, 0.1, and 0.5 saturation with magnesium sulphate. The precipitate was separated from the supernatant by centrifuging at 9,500 g. for 20 minutes, after the solution had been kept in the cold room for a few hours. The supernatant was kept overnight at 3° C., and the next fraction precipitated the following day.

Subfractions were obtained in a similar manner, but to distinguish them from the main fractions, different nomenclature was used. Main fractions were labelled as I, II, III, IV and V, whereas the subfractions obtained at the same concentration of magnesium sulphate was labelled as A, B, C, D, and E respectively.

Protein samples were hydrolysed by refluxing them for 24 hrs. with 20% hydrochloric acid.

Total nitrogen determinations were carried out using the micro-Kjeldahl method (89), using $HgSO_4$ and K_2SO_4 as catalyst. The ammonia was collected in 4% boric acid and titrated with standard sulphuric acid using methylene blue-methyl red indicator. Amide nitrogen determinations on the acid hydrolysates were carried out by a modification of the above method. Aliquots of 10 ml. of the acid hydrolysate were distilled with 10 ml. of the alkali, the ammonia evolved being collected in 4% boric acid and titrated with standard sulphuric acid.

Sedimentation velocity experiments were carried out using a Spinco Model E ultracentrifuge equipped with phase plate and electronic temperature control unit, at about 60,000 r.p.m., equivalent to 250,000 g., at 20° C. Some experiments, however, were done before the temperature control unit was installed so that corrections had to be made for these runs.

The sedimentation coefficients were calculated according to the formula (90),

$$S = \frac{2.303\ (\log x_2 - \log x_1)}{w^2(t_2 - t_1)},$$

where S = sedimentation coefficients in Svedberg units (Svedberg unit = 1 x 10^{-13} sec.),

x_2 = distance in cm. of peak from center of rotation at time t_2,

x_1 = distance in cm. of peak from center of rotation at time t_1,

w = speed of the rotor in radians per second,

t_2 and t_1 = time in seconds at time of measurement.

The values obtained from successive pairs of diagrams were averaged to obtain the observed sedimentation coefficient for the experiment. Observed sedimentation coefficient (S_{obs}) was converted to a standard value according to the formula (90),

$$S_{20,w} = S_{obs} \cdot \frac{\eta_t}{\eta_{20}} \cdot \frac{\eta}{\eta_{20}} \cdot \frac{(1 - \bar{V}\ \rho_{20,w})}{1 - \bar{V}\ \rho_t},$$

where $S_{20,w}$ = sedimentation coefficient in Svedberg units at 20° C. in water,

$\frac{\eta_t}{\eta_{20}}$ = viscosity of water at temperature t relative to that at 20° C.,

$\frac{\eta}{\eta_{20}}$ = relative viscosity of solvent at 20° C.,

$\rho_{20,w}$ = density of water at 20° C., in gm. per ml.,

ρ_t = density of solvent at temperature t, in gm. per ml.,

$\bar{V}$ = partial specific volume in gluten in the particular solvent, in ml. per gm.

The $\frac{\eta_t}{\eta_{20}}$ values were taken from Svedberg and Pederson (90). The relative viscosities of the two solvents used in this study were determined at 20° C. with an Ostwald's viscometer and were found to be 1.196 and 1.035 respectively for 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml. and for 0.02 M aluminum lactate buffer, pH 3.05. Corresponding densities of the solvents at 20° C. were found to be respectively 1.032 and 1.001 gm. per ml.

Partial specific volume for gluten in sodium salicylate is 0.700 ml. per gm. (57), and for the gluten in 0.02 M aluminum lactate buffer, pH 3.05 was found to be 0.706 ml. per gm. (see Appendix).

A Beckman model G pH meter was used to determine the hydrogen ion concentrations.

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A Beckman model G pH meter was used to determine the hydrogen ion concentrations.

EXPERIMENTAL RESULTS

A. Fractionation of gluten dispersed in sodium salicylate containing sodium bisulfite

(i) Effect of gluten concentration on the yield of various fractions

McCalla and associates (55, 56, 57) stated that the number of fractions which can be obtained from gluten can be varied by varying one or more conditions of fractionation. Jones *et al.* (64) were unable to fractionate gluten dispersed in aluminum lactate buffer if the concentration of gluten was as high as 2%. This observation suggested that the concentration of gluten in the dispersion might have some effect on the yield of the fractions. As there is no information available in the literature, an attempt was made to obtain some.

Gluten dispersions containing different amounts of protein were prepared in the usual manner in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml. Five fractions were obtained from each dispersion by the successive additions of the requisite volumes of saturated magnesium sulphate solution. The details of the procedure followed have already been described in the section on Materials and Methods.

The fractions obtained from gluten dispersions were redispersed in 8% sodium salicylate, except for fraction I which could not be redispersed. McCalla and Rose (55) were able to redisperse fraction I by grinding it in a mortar with sodium salicylate. However, no effort was made to dissolve this fraction in a similar manner. Other fractions dispersed easily, the rapidity of dispersion increasing with the solubility of the fractions. The resulting solutions differed from each other in turbidity, the redispersion of fraction II being the most turbid while that of fraction V was clear.

Results presented in Table I show that the yield of the various fractions differs if the concentration of proteins in the dispersions is different. The amount of protein precipitated as fraction II shows a marked dependence on concentration of gluten in the dispersion. Over 70% of the protein is precipitated as Fraction II where the concentration of protein is as high as 5.5%, while only about 33% is precipitated if the gluten concentration is about 2.15%. This variation is reflected in other fractions also, but no definite statements can be made on the basis of the results. However, the fact that about 75% of the protein can be precipitated as the rather insoluble second fraction, indicates that precipitation techniques are not very satisfactory in work designed to isolate definite protein entities.

Table I. The effect of protein concentration of the dispersion on the yield of different fractions

Protein concentration in dispersion, %	Yield of fraction as % of total					Total recovery, %	Amide-nitrogen as % of total nitrogen				
	I	II	III	IV	V		I	II	III	IV	V
1.13	*	34.4	27.9	11.9	20.6	94.8+		23.7	25.5	27.2	24.7
2.01	1.2	34.2	28.1	11.7	21.8	97.0	15.2	23.9	29.3	25.8	24.0
2.15	18.1	33.2	14.9	11.5	19.6	97.3	19.7	23.3	24.7	25.0	25.0
4.25	15.0	49.1	15.4	7.2	17.8	104.5	20.0	24.0	24.0	25.3	23.1
5.58	2.8	71.3	8.9	4.2	11.6	98.8	18.1	24.3	25.5	25.9	22.6
6.19	4.8	71.1	11.7	5.7	16.9	110.2	16.5	21.9	25.3	24.2	22.5

*The amount of protein precipitated was not estimated.

The variations in the yields of fraction I occured under conditions apparently unrelated to the gluten concentration. Careful control of all the variables should provide additional information in this regard. Most of the other variations can be largely accounted for by the differences in fraction II. Since fractionation was not the major field of this investigation, no attempt was made to repeat the experiment.

It was considered desirable, however, to use as constant conditions as possible for all similar experiments.

In general the amide-nitrogen content of the fractions increased as their solubility increased, except in the case of fraction V. McCalla and Rose (55) had obtained similar results for the most soluble fraction and have explained them by showing that this most soluble fraction apparently contains the soluble non-gluten proteins of wheat flour, which were not removed completely during washing of gluten and which are low in amide-nitrogen. Nearly all amide-nitrogen results agree in general with those already published (55, 57). Amide-nitrogen content of fractions I and V showed definite increases if the amount of protein precipitated in the fraction increased. Both these fractions are known to be low in amide-nitrogen. An increase in the amount of nitrogen precipitated as

fraction I means, that some of the higher amide-containing protein was included in this fraction. Because of this an increase in the total amide-nitrogen content would be expected. Similarly, fraction V contains non-gluten proteins low in amide-nitrogen content. As the amount of protein precipitated as fraction V increases, the amount of gluten-protein precipitated together with non-gluten protein increases, resulting in higher amide nitrogen content.

(ii) Refractionation of gluten fractions

An experiment was conducted in which the gluten fractionsobtained in the usual manner were redispersed in 8% sodium salicylate solution and refractionated by the addition of the requisite volumes of saturated magnesium sulphate solution. The number of subfractions obtained from each fraction varied. Five being the number which were obtained from fraction II but only two were obtained from fraction V. Fraction I, as already stated, could not be redispersed, hence was not refractionated.

The results presented in Table II might appear to contradict the contention of McCalla and associates (55, 56, 57) that the successive fractions obtainable from gluten vary progressively. In every case a portion of the protein has precipitated at lower concentrations of magnesium

Table II. Fractionation of gluten dispersion in sodium salicylate with sodium bisulfite containing 2.19% protein

Material	$S_{20,w}$	Yield of the fraction as % of gluten	Amide nitrogen as % of total-N
Gluten	1.93		23.8
Fraction I		2.6	16.9
Fraction II	2.22	52.9	23.8
Fraction III	2.06	19.7	24.9
Fraction IV	2.05	7.4	27.1
Fraction V	1.77	14.5	23.5
Total		97.1	
		Yield of the subfraction as % of fraction	
Fraction II			
Subfraction A	2.23	36.0	23.1
Subfraction B	2.34	43.4	24.5
Subfraction C	2.18	12.8	25.4
Subfraction D	2.08	5.5	26.1
Subfraction E	2.01	6.3	25.4
Total		104.0	
Fraction III			
Subfraction B	2.14	21.3	23.6
Subfraction C	2.16	43.9	25.4
Subfraction D	2.07	17.2	*
Subfraction E	2.01	18.6	*
Total		101.0	
Fraction IV			
Subfraction C	2.15	23.6	*
Subfraction D	2.16	37.2	27.6
Subfraction E	2.04	38.5	*
Total		99.3	
Fraction V			
Subfraction D	2.02	14.6	25.8
Subfraction E	1.76	82.5	23.1
Total		97.1	

*Samples lost

sulphate than that at which the original fraction was precipitated. In fractions II, III, and IV, a considerable proportion of the total protein has precipitated at higher concentrations of magnesium sulphate than that at which the fractions were precipitated. These observations might be considered to support the view that gluten is a protein complex consisting of a number of components (55, 56, 57). If the fractions were individual proteins, the total protein present in them should have precipitated at the same concentration of magnesium sulphate as that at which they were originally precipitated. Except in the case of fraction V, only about 80% of the total protein is precipitated by that particular concentration of magnesium sulphate. This amount includes protein precipitated at lower concentration of magnesium sulphate.

It is quite possible that some loss of solubility may be attributed to the rather extensive handling involved in redispersions. McCalla and Gralén (57) found gluten very sensitive to handling. It is also likely that the apparent increase in solubility of parts of each fraction is the result of failure to get clear-cut separation on the original precipitation.

The sedimentation coefficients of the fractions and subfractions did not show a well-defined decrease with increase in their solubility although there was a tendency

towards this. Such a decrease was reported in earlier work (57).

The amide-nitrogen content of the main fractions followed a trend similar to that found by McCalla and Rose (55). Complete information is available only for subfractions from fractions II and V and these exhibit the expected trends. Amide-nitrogen values for the two most soluble fractions of fraction III and for two subfractions from fraction IV are missing because samples were lost, hence a complete appraisal is not possible. It is clear, however, that all of the analytical results that are available agree with those obtained earlier.

In view of the secondary nature of these investigations in the whole project no effort was made to obtain the missing results by repeating the experiment.

B. Preliminary sedimentation and enzymic hydrolysis experiments

Before investigating the enzymic hydrolysis of wheat gluten it was necessary to determine the suitability of the dispersing agents for this study. The desired characteristics of a dispersing medium were its ability to disperse gluten completely and without any change in its character, to lend itself to sedimentation studies and to

provide a medium in which the enzymes would act on gluten. Sodium salicylate has been shown (77, 57) to possess the first two of the three characteristics. Jones et al. (64) have dispersed gluten in 0.02 M aluminum lactate buffer, pH 3.1; and Swan's reagent (87) has been claimed to solubilise wheat gluten completely (86). Sodium salicylate solution containing bisulfite had been used in some earlier work in this laboratory and shown to reduce the time of dispersion from 5 - 6 hours to 2 hours. Gluten obtained from wheat flour according to the procedure outlined earlier was dispersed in these media and subjected to sedimentation analysis. The sedimentation diagrams in Fig. 1 show that gluten dispersed in each of these media appears to be in essentially the same physical state in all of them. All diagrams show only one peak. The shape of the peaks obtained with gluten dispersed in sodium salicylate with and without bisulfite are very much alike even though the reducing agent is reported to cause changes in physical properties of gluten, and also causes considerable changes in the chemical properties of many proteins (91). The diagrams for other media are different from those for the salicylate media only so far as the base line is concerned. In the case of salicylate media there is evidence of displacement of solvent molecules at the top of the cell, whereas in the case of the other two media the base line is almost horizontal. This difference is caused by the

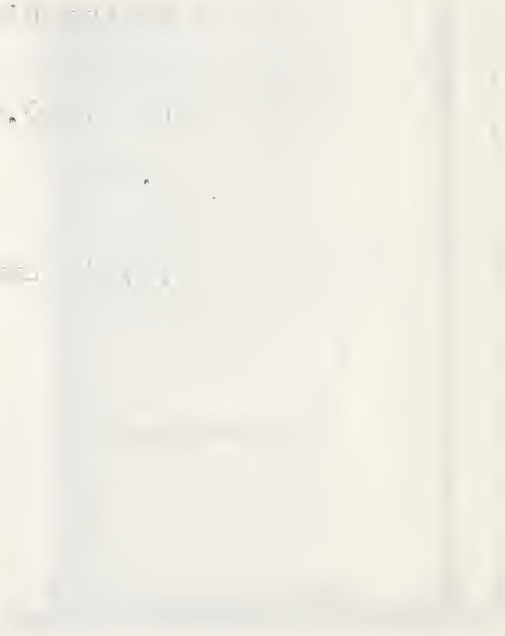


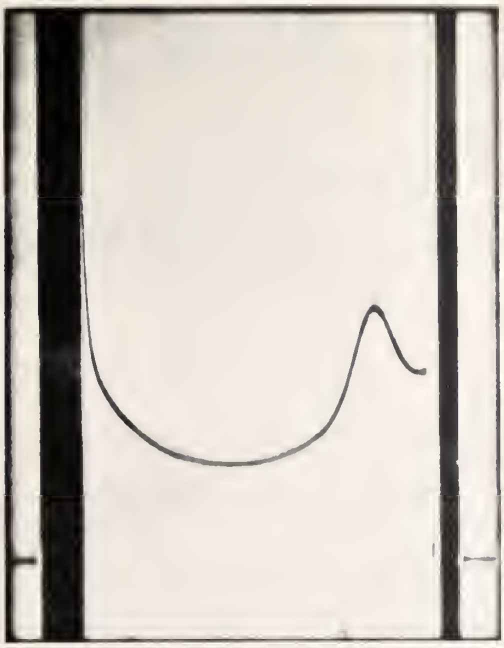
Figure 1. Sedimentation diagrams of gluten, after 89 mins. at 250,000 g.

(a) in 8% sodium salicylate

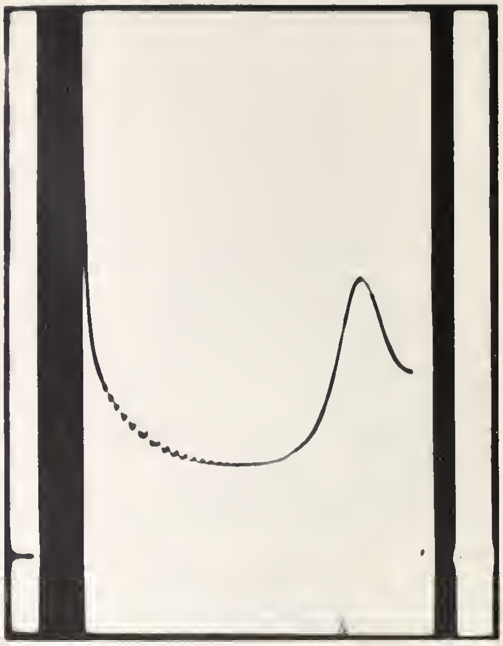
(b) in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml.

(c) in 0.02 M aluminum lactate buffer, pH 3.05

(d) in Swan's reagent



(a)



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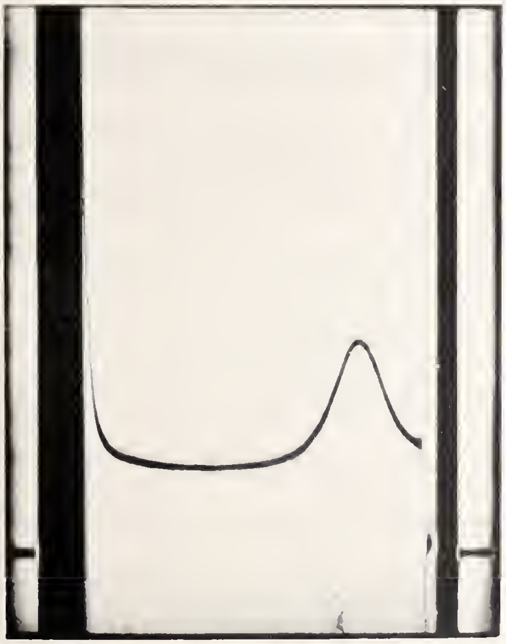
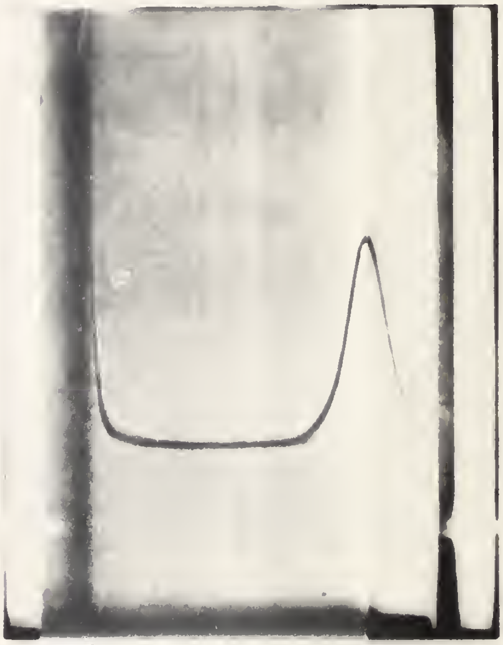


(c)



(d)

high concentration of sodium salicylate in these solvents, a condition that has been shown to yield poor base lines.

A consideration of the sedimentation coefficient obtained for gluten in each of the four media shows that there is little difference in the molecular size of gluten as dispersed in these. $S_{20,w}$ values for gluten in 8% sodium salicylate, in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml., and in 0.02 M aluminum lactate buffer pH 3.05, were 2.04, 2.09, and 1.90 respectively. The relatively small differences between the $S_{20,w}$ values do not represent any really significant change in the molecular size. Experiments conducted with varying concentrations of gluten have shown that the sedimentation coefficient is somewhat concentration dependent, though this effect is not very marked in the range of concentrations used. Some experiments were also conducted before the electronic temperature control unit was installed. In those cases the correction for temperature is at best approximate. In view of these facts and of earlier repetition on individual preparations it seems reasonable to assume that differences of the order of 0.1, or perhaps even 0.2 Svedberg units in the sedimentation coefficients do not reflect real differences in the molecular size under the conditions of these experiments.

The observed sedimentation coefficient for gluten in Swan's reagent was 1.61 at 20° C. This compares roughly with the corresponding value of 1.75 for gluten in aluminum lactate. The difference does not seem to be an indication of any major difference in molecular size of the gluten in the two media. Correction of the observed sedimentation coefficient for gluten in cupric-ammonium-sulfite, to $S_{20,w}$ would have involved determinations of viscosity and partial specific volume values. As the reagent was not used in the enzyme investigations, these necessary values for correction of observed sedimentation coefficient to $S_{20,w}$ were not determined.

McCalla and Gralen (57) discussed the sedimentation characteristics of gluten in 8% sodium salicylate. The results obtained in the present study are similar to those obtained by them, hence it can be concluded that so far as sedimentation behaviour is concerned, the other dispersing agents can be used with equal assurance.

Having shown that all the dispersing agents possess two of the three requisites, the next step was to find out whether the proteolytic enzymes would act on gluten dispersed in them. The pH requirements of the enzymes appeared to make it clear that Swan's reagent (87) could not be used in any of the experiments as none of the enzymes would act

normally in such an alkaline medium. Therefore, only three dispersing media could be used in enzymic studies, two of these being salicylate media and the third, the aluminum lactate buffer (64). On the basis of the pH of the media and the range in which the enzymes are active, sodium salicylate and sodium salicylate containing sodium bisulfite should be suitable for experiments with papain and the neutral protease of Fermex MT, while aluminum lactate buffer should be suitable for pepsin and the acid protease of Fermex MT.

Results of the experiments conducted with papain and Fermex MT on gluten dispersed in 8% sodium salicylate, and 8% sodium salicylate containing sodium bisulfite are presented in Fig. 2 and 3. It is clear from the shape of the sedimentation diagrams that Fermex MT acted on gluten dispersed in both solvents, whereas papain acted only in sodium salicylate containing sodium bisulfite. Where the enzyme was active it seems quite clear that variable-sized products have been produced by the enzymes.

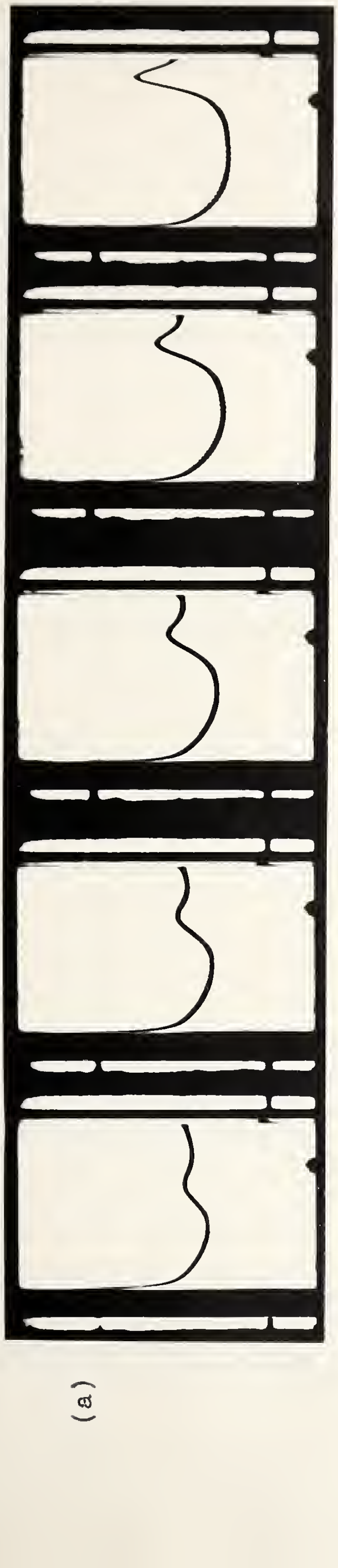
Since papain required the presence of a reducing agent for its full activity (92, p. 285), it may be in an inactive state in the sodium salicylate dispersion, but in an active state in sodium salicylate containing sodium bisulfite.

Figure 2. Effect of enzymes on gluten dispersed in sodium salicylate. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

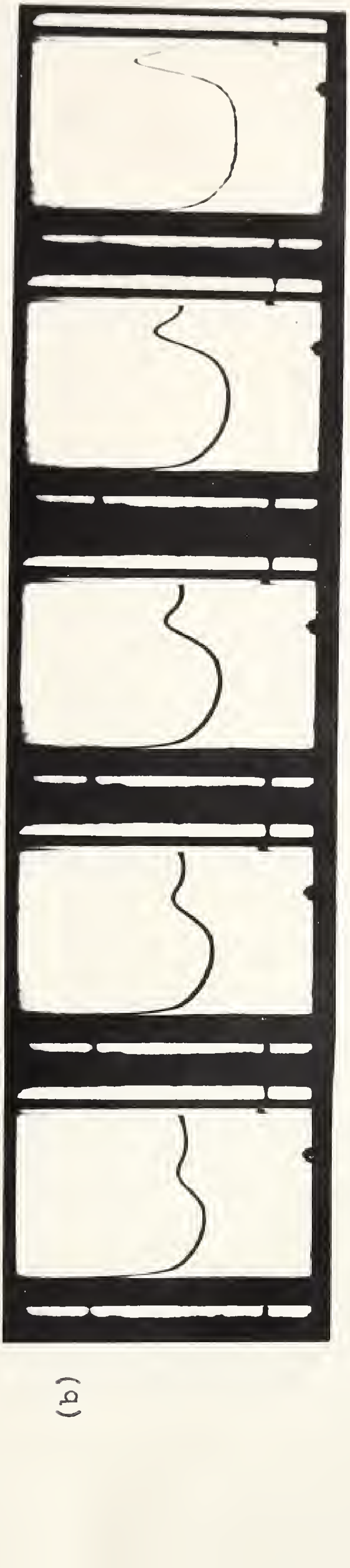
(a) control

(b) plus 2 mg. papain per 100 ml., for 12 hrs. at 37° C.

(c) plus 100 mg. Fermex **MT** per 100 ml., for 12 hrs. at 37° C.



(a)



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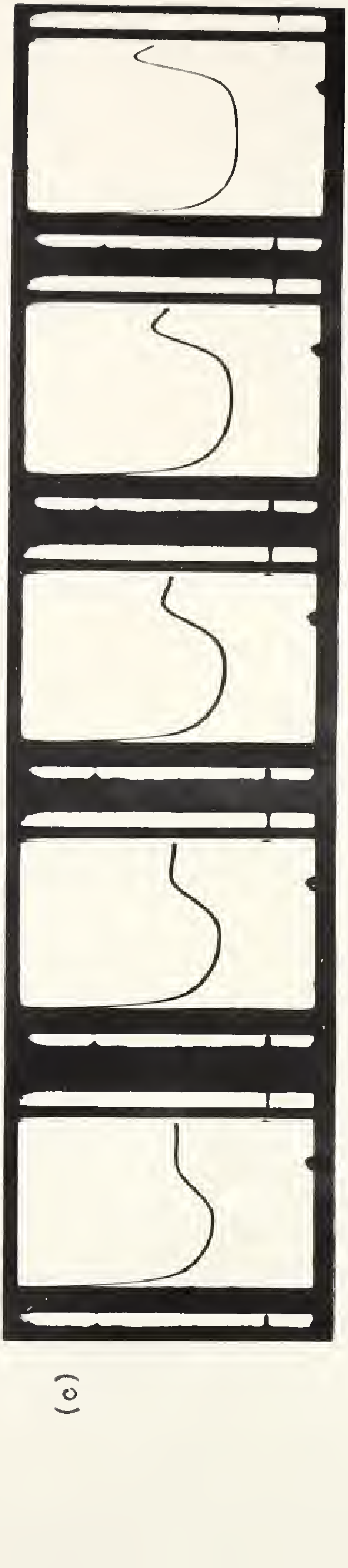


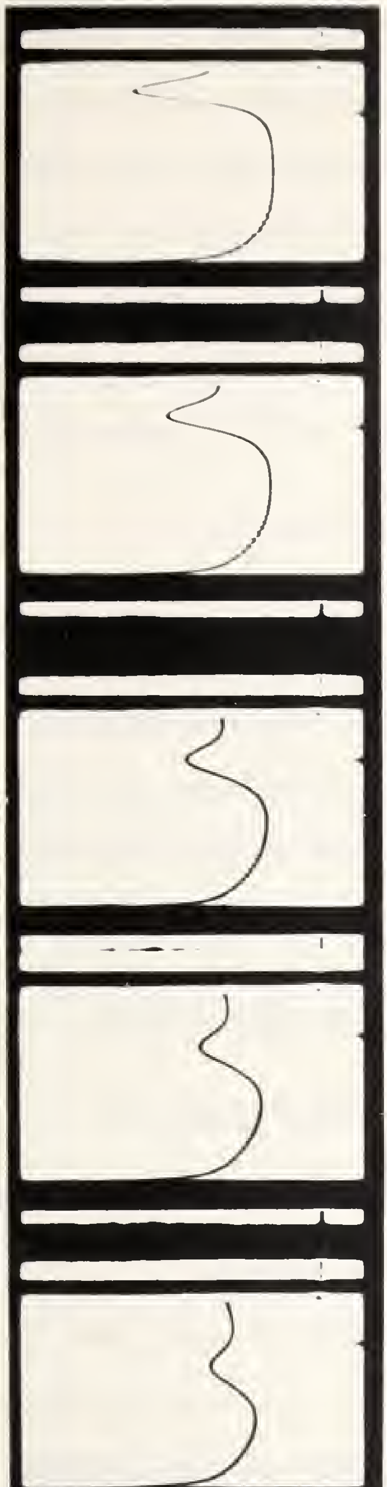
(c)

Figure 3. Effect of enzymes on gluten dispersed in sodium salicylate containing sodium bisulfite. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

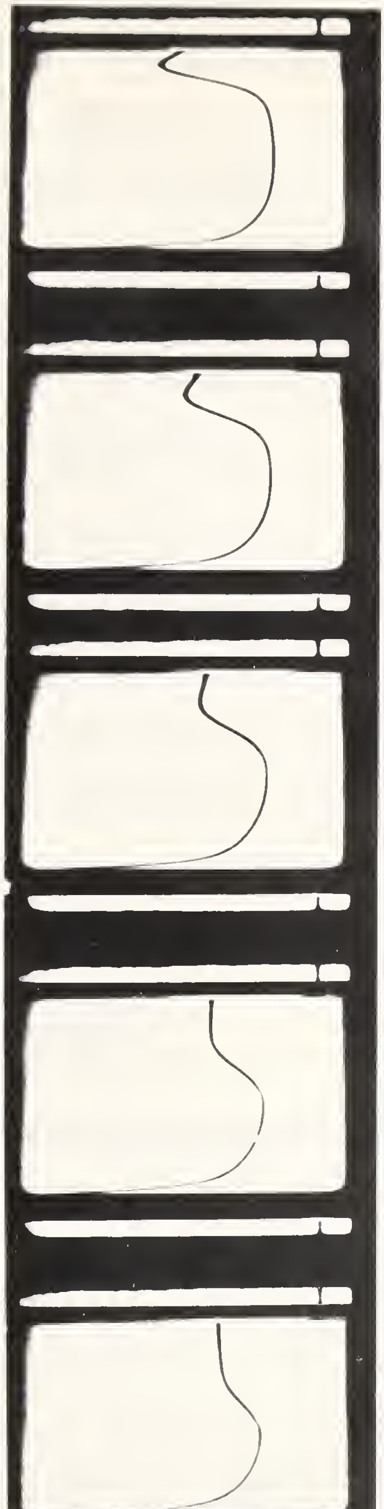
(a) control

(b) plus 2 mg. papain per 100 ml., for 12 hrs. at 37° C.

(c) plus 100 mg. Fermex MT per 100 ml. for 12 hrs. at 37° C.



(a)



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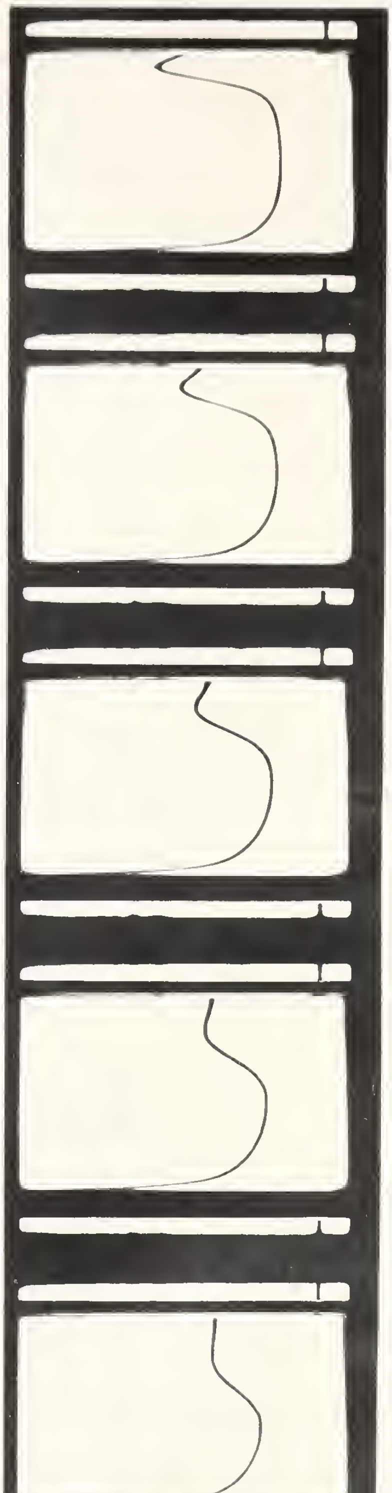


(c)

Experiments with pepsin and the acid protease of Fermex MT were carried out on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05. The shape of the sedimentation diagrams of these experiments (Fig. 4) although quite different from those in Fig. 2 and 3 show that both the enzymes act on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05. The effects of the enzymes are discussed in the following sections.

On the basis of the above observations, sodium salicylate containing bisulfite was used for experiments with papain and the neutral protease of Fermex MT, whereas aluminum lactate buffer was used for experiments with pepsin and the acid protease of Fermex MT. Experiments on gluten fractions with Fermex MT were carried out in 8% sodium salicylate.

C. Experiments with Fermex MT

(i) Experiments on gluten dispersed in 8% sodium salicylate containing sodium bisulfite

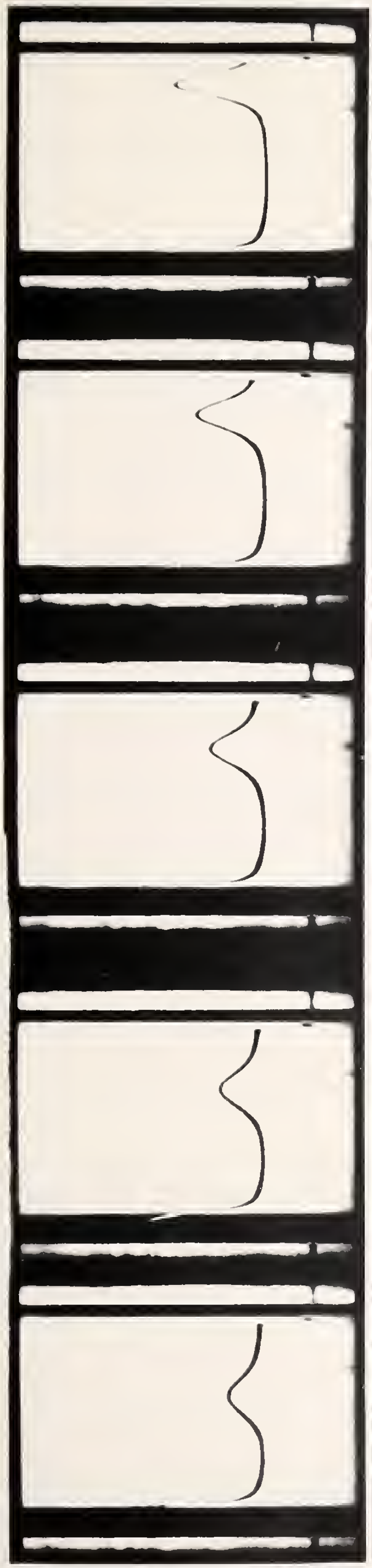
Fermex MT was added to gluten dispersion at 37° C., at the rate of 100 mg. per 100 ml. Aliquots were removed at intervals of 4, 12, and 36 hours for sedimentation analyses. Results of the experiments are presented in Fig. 5 and Table III.

Figure 4. Effect of enzymes on gluten dispersed in aluminum lactate buffer. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

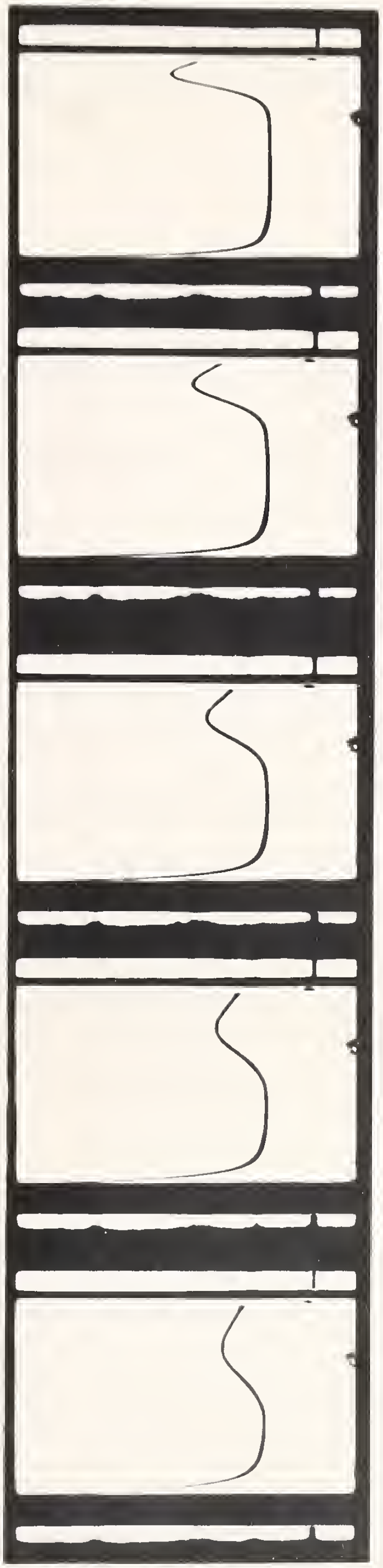
(a) control

(b) plus 20 mg. pepsin for 100 ml., for 12 hrs. at 25° C.

(c) plus 100 mg. Fermex MT per 100 ml., for 12 hrs. at 25° C.



(a)



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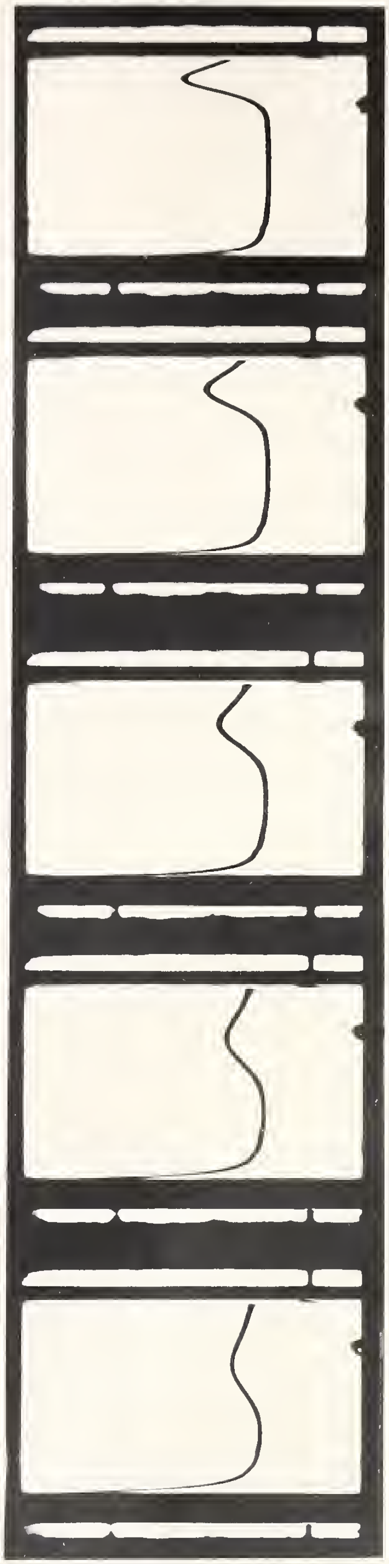


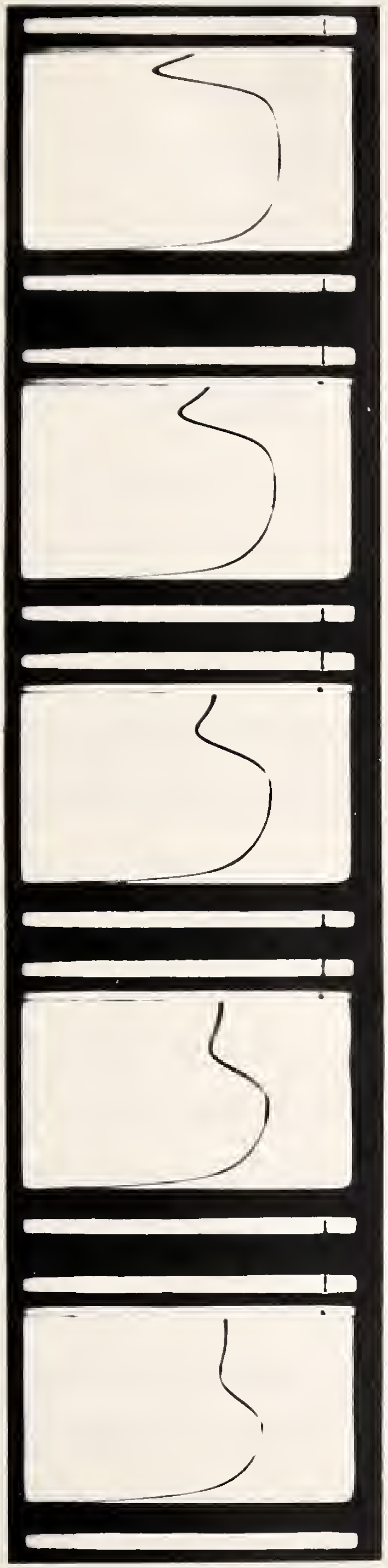
(c)

Figure 5. Effect of Fermex MT, at 100 mg. per 100 ml., in gluten dispersed in sodium salicylate containing sodium bisulfite. First photograph after 57 mins. Others at intervals of 32 mins.

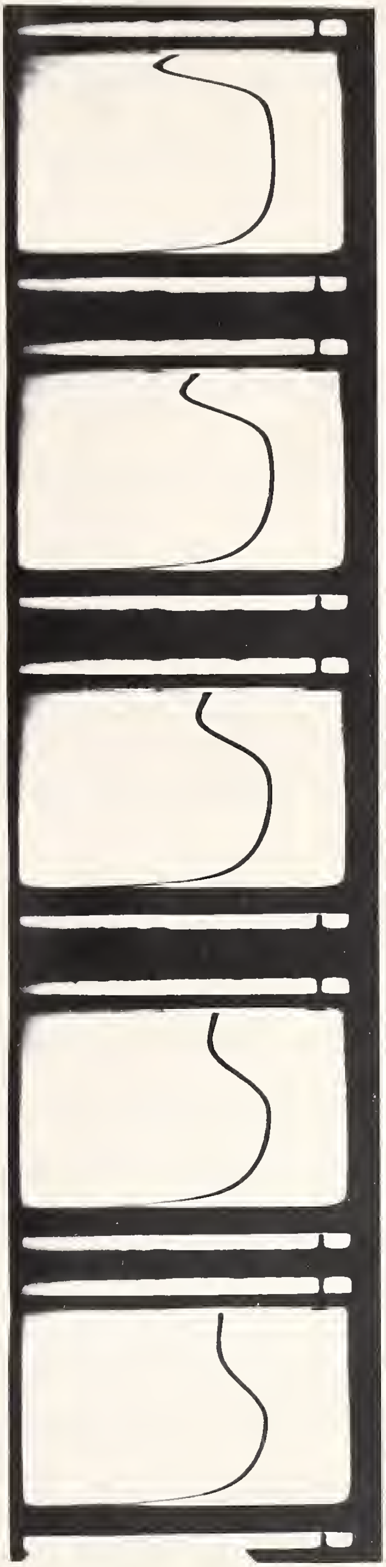
(a) for 4 hours at 37° C.

(b) for 12 hours at 37° C.

(c) for 36 hours at 37° C.



(a)



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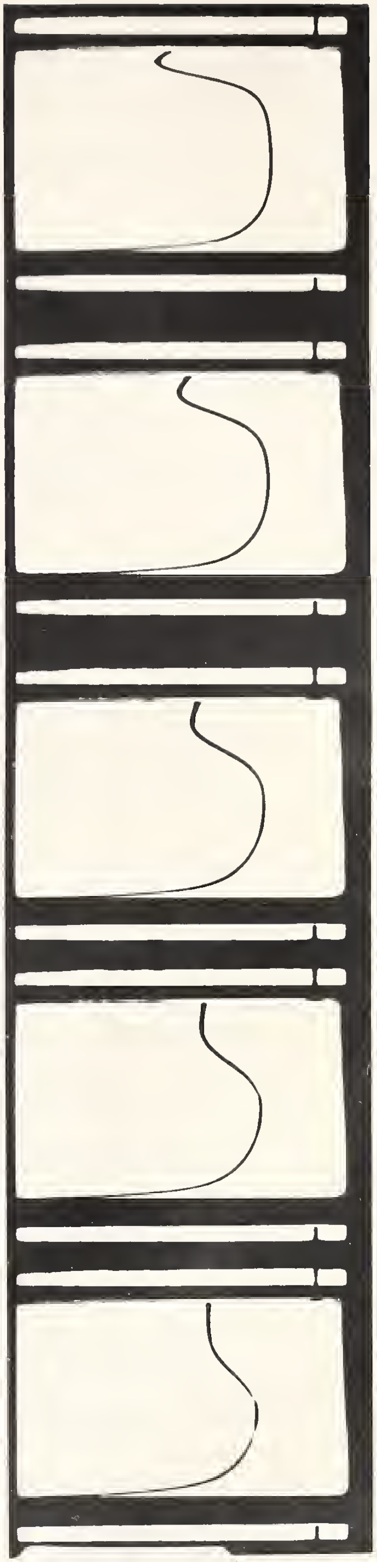


(c)

Table III. Effect of Fermex MT at 100 mg./100 ml. on gluten dispersed in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml. dispersion, at 37° C. Sedimentation coefficient of original gluten dispersion=2.11

Time of reaction	Sedimentation coefficient			Protein pptted.*
	after digestion	redispersed after digestion	simultaneously dialysed during digestion	
4 hours	1.59	1.85		67.9
12 hours	1.31	1.46	1.51	60.0
36 hours	0.90	1.37	1.50	42.2

*Amount of protein precipitated by half saturation of enzymic digests with magnesium sulphate, percent of total protein

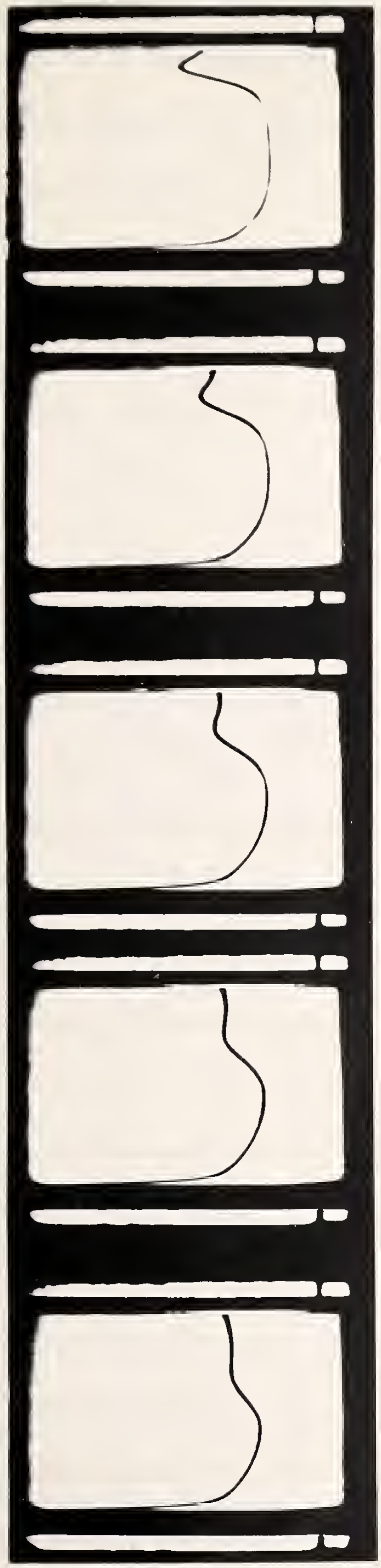
The table shows that the sedimentation coefficient of the enzymic digest decreased as the reaction time increased. The differences in the shape of the sedimentation diagrams are less marked although the shape after 4 hours is distinctly different from that after the longer periods. The rate of movement of the boundary decreased as the reaction time increased. These observations show clearly that the mean molecular size of the protein was decreased by enzyme action. The characteristic 'sickle' shape of the diagrams indicates that the enzyme produces substances of variable molecular weight which sediment at different but much slower rates than the original gluten molecule.

In order to measure the extent of hydrolysis of gluten and to separate the protein material from non-protein nitrogenous compounds produced during enzymic digestion, the enzymic digest was brought to half-saturation with magnesium sulphate. The precipitate was separated by centrifuging at 9,500 g. for 20 minutes, and redispersed in 8% sodium salicylate. The results of the sedimentation runs on the redispersions are presented in Fig. 6 and Table III. The shapes of sedimentation diagrams do not appear to differ from those in Fig. 5, but the sedimentation coefficients were higher than those obtained with the original enzymic digests. The increase in the sedimentation coefficients indicates a partial removal of the products of hydrolysis. Progressive decrease in the amount of protein precipitated by half-saturation of the enzymic digests with magnesium sulphate shows increased hydrolysis of gluten as the time of reaction was increased.

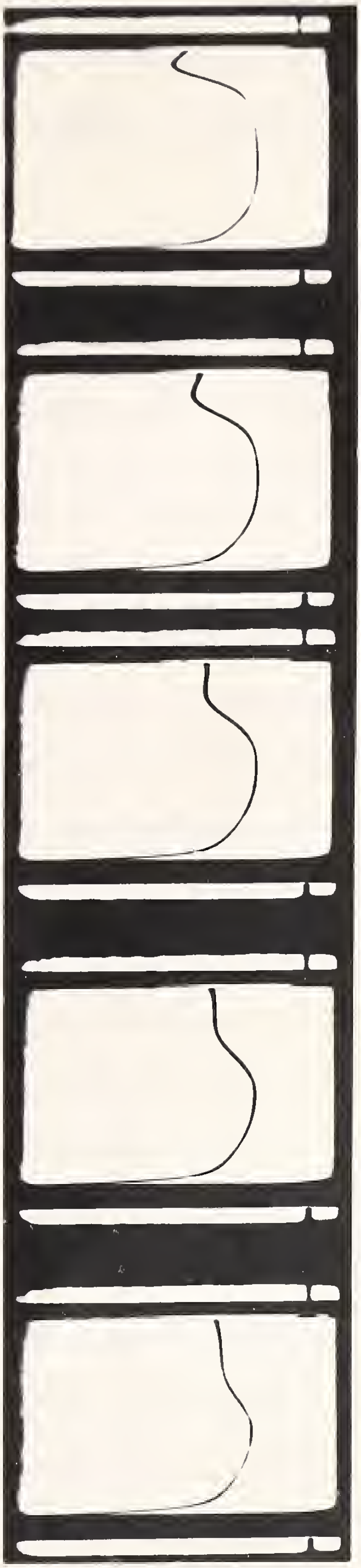
Craig and colleagues (93) have separated proteins and peptides by dialysis. This method was also used, in the present study, for the removal of the products of hydrolysis. However, as dialysis of the enzymic digests following the reaction-period would have greatly increased the time of reaction, continuous and simultaneous dialysis was used in the present investigations. Aliquots were taken for sedimentation runs after 12 and 36 hours. The

Figure 6. Effect of precipitation and redispersion on Fermex MT digests. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) after 4 hours action at 37° C.
(b) after 12 hours action at 37° C.
(c) after 36 hours action at 37° C.



(a)



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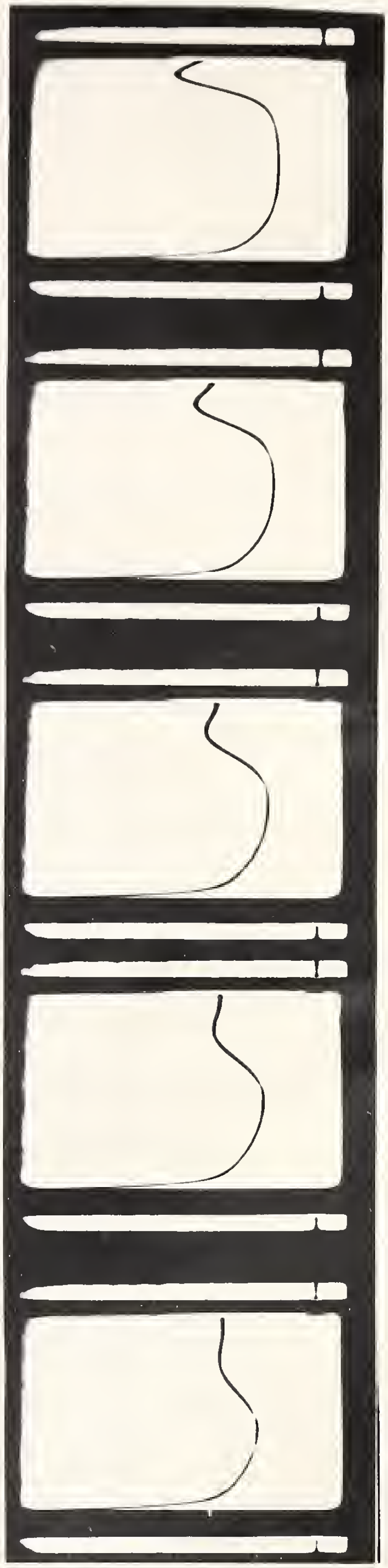


(c)

results (Table III) show that there was a slight increase in the sedimentation coefficients of the enzymic digest. The peaks in the sedimentation diagrams (Fig. 7) show some sharpening.

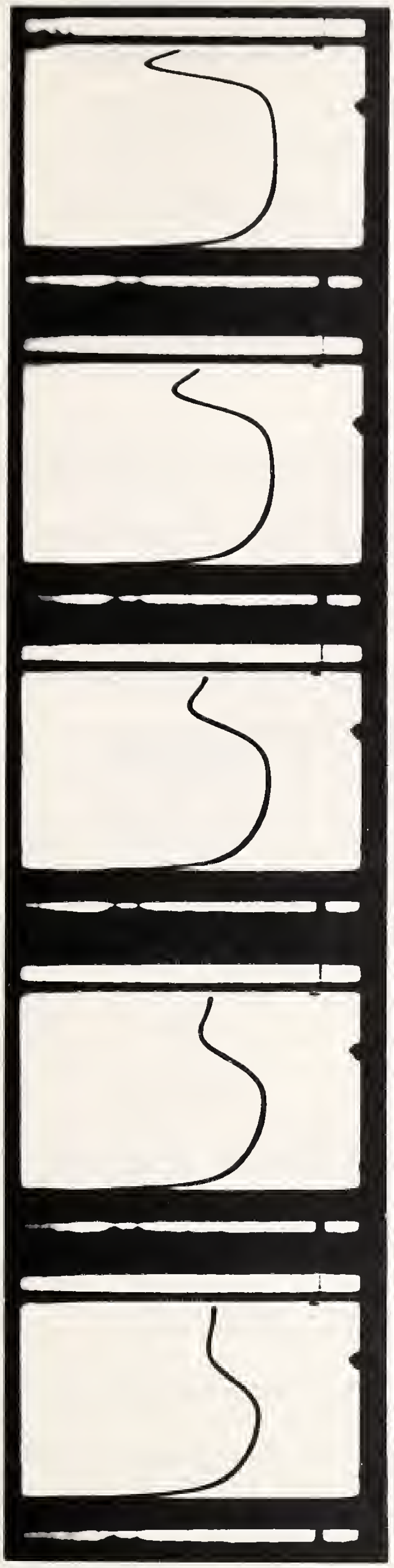
A bulky precipitate was formed in both the dialysis experiments and was removed before sedimentation analysis. This material is discussed later in the thesis.

A consideration of the results presented in this section shows that the neutral protease of Fermex MT readily hydrolyses gluten. During 36 hours of enzymic digestion about 58% of the total protein is changed to substances which cannot be precipitated by half saturation with magnesium sulphate. The precipitated protein has a much lower sedimentation coefficient than the original gluten molecule, indicating that the mean molecular size of the precipitated protein is considerably smaller than that of gluten. Even though the gluten molecule is broken into substances of variable molecular size, the sedimentation diagrams show only one peak, suggesting that there is a continuous spectrum of products of hydrolysis. As the specificity of the enzyme is not known no statement about the pattern of hydrolysis can be made. However, it seems certain that the neutral protease of Fermex MT must hydrolyse many types of peptide bond.

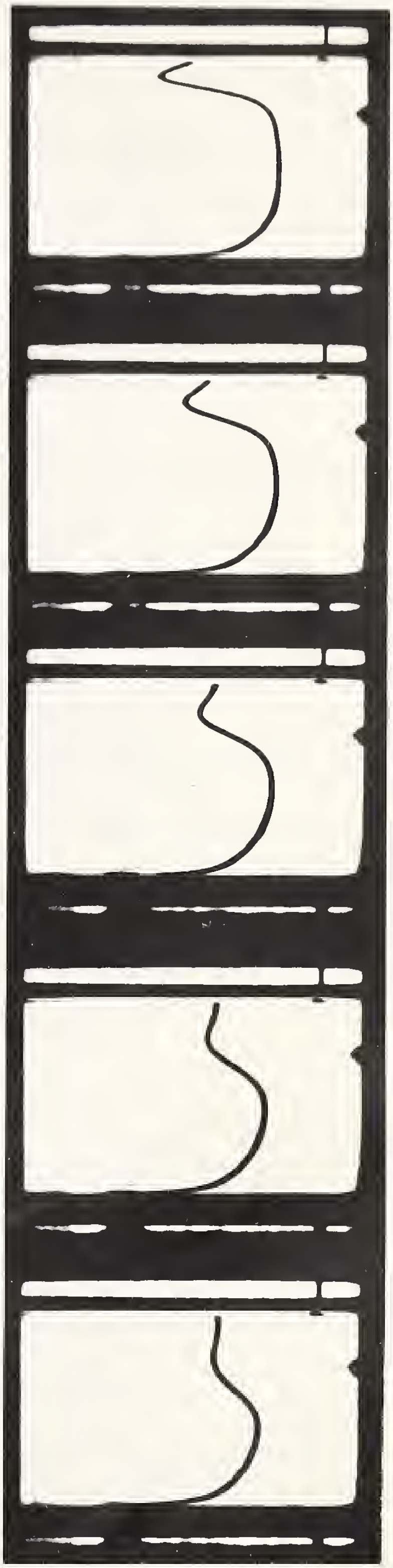
Figure 7. Effect of simultaneous dialysis on Fermex MT digests. First photograph after 57 mins., at 250,000 g. Others at invervals of 32 mins.

(a) during 12 hours action at 37° C.

(b) during 36 hours action at 37° C.



(a)



(b)

(ii) Experiments on gluten fractions redispersed in 8% sodium salicylate

Experiments were carried out on gluten fractions II, III, IV, and V treated in the manner described above, allowing the enzyme to act for 12 hours at 37° C. The results are presented in Fig. 8 and Table IV.

Table IV. Effect of Fermex MT at 100 mg./100 ml. on gluten fractions redispersed in 8% sodium salicylate. Reaction time 12 hours at 37° C.

Material	Sedimentation coefficient			Protein precipitated*
	before digestion	after digestion	redispersed after digestion	
Fraction II	2.22	1.67	1.58	66.9
Fraction III	2.06	1.25	1.45	78.5
Fraction IV	2.05	1.25	1.32	81.1
Fraction V	1.77	1.38	1.43	81.0

*Amount of protein precipitated by half saturation of the enzymic digests with magnesium sulphate, percent of total protein

It is clear from the table and the figure that the action of the neutral protease of Fermex MT on gluten fractions is somewhat similar to that on the whole gluten. Sedimentation coefficients of the enzymic digests are considerably lower

Figure 8. Effect of Fermex MT, at 100 mg./100 ml. on gluten fractions redispersed in sodium salicylate. Photographs after 153 mins. at 250,000 g.

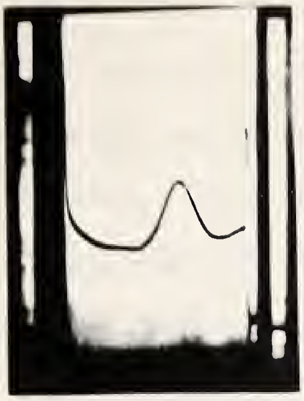
(a) (i) fraction II
  (ii) fraction II plus Fermex MT, for 12 hours at 37° C.
  (iii) precipitated and redispersed

(b) (i) fraction III
  (ii) fraction III plus Fermex MT, for 12 hours at 37° C.
  (iii) precipitated and redispersed

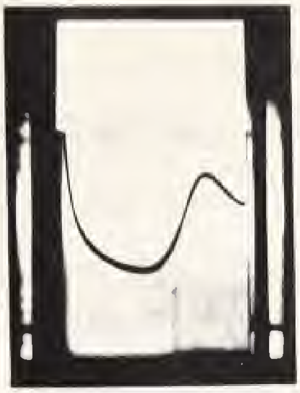
(c) (i) fraction IV
  (ii) fraction IV plus Fermex MT, for 12 hours at 37° C.
  (iii) precipitated and redispersed

(d) (i) fraction V
  (ii) fraction V plus Fermex MT, for 12 hours at 37° C.
  (iii) precipitated and redispersed

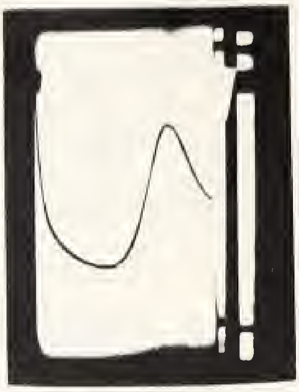
(a)
(i)



(a)
(ii)



(a)
(iii)



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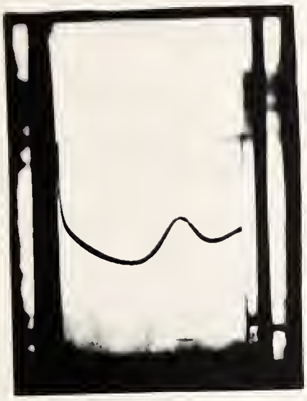

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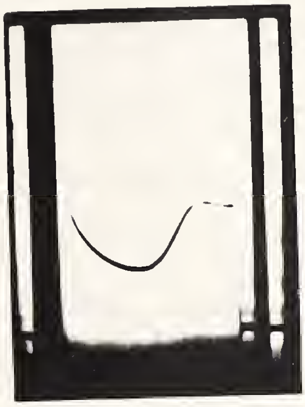

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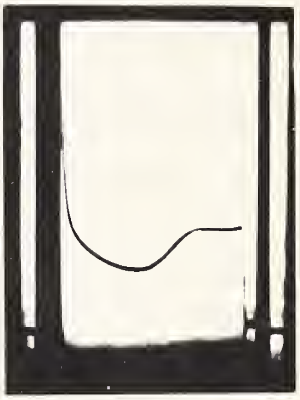
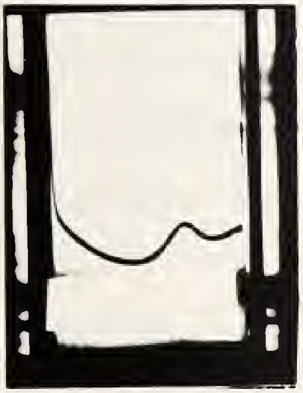


(c)
(i)



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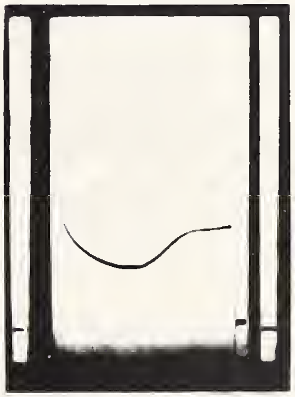

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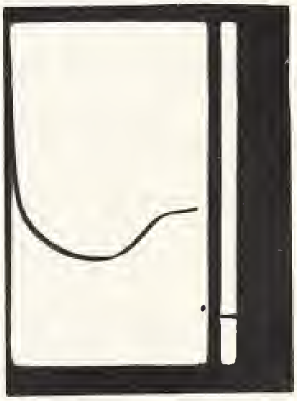
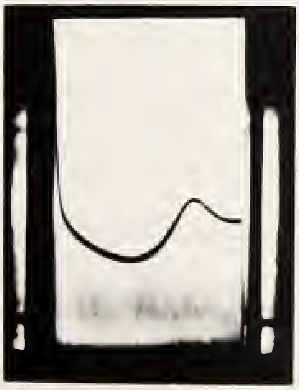
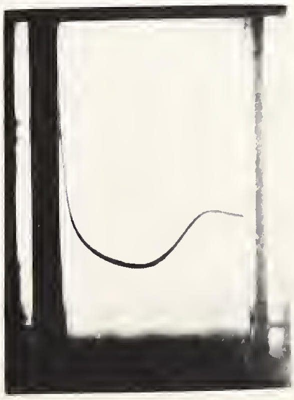


(d)
(i)



(d)
(ii)



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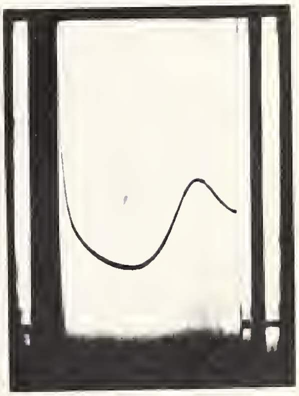


than those of the original dispersions. Except in fraction II, there is an increase in the sedimentation coefficient of the redispersed material over that of the enzymic digests. However, the increase in the values may not be significant. The shape of sedimentation diagrams for the protein precipitated by half saturation of the enzymic digests with magnesium sulphate suggests that the action of the enzyme on fractions III and IV was different from that on fractions II and V. A strict comparison is not possible, however, as the protein concentrations in the redispersions were not the same. They were much lower for fractions III and IV, being respectively 0.67 and 0.48%, whereas the concentrations of the protein in the redispersions of fractions II and V were respectively 1.36 and 1.69%. A better idea of the rate of hydrolysis is given by the amounts of protein precipitated by half saturation of the enzymic digests with magnesium sulphate. Except for fraction II, the amount of protein precipitated was approximately the same, about 80%. For fraction II it was much lower, being about only 67%. This suggests that the more soluble fractions of gluten are digested less than the more insoluble fraction. In spite of the differences in the protein concentration of the redispersions of the fractions the shape of the diagrams does suggest differences in the pattern of hydrolysis. The shape of the sedimentation diagram for the enzymic digest of fraction II suggests that a much smaller proportion of low molecular

weight material was produced whereas those of the others assumed the characteristic 'sickle' shape indicating the presence of a greater proportion of low molecular weight substances. This difference in the pattern of hydrolysis is shown most clearly by the sedimentation diagrams for the redispersed material. In the case of fraction II, the boundary left the meniscus, whereas the boundaries in the diagrams for fractions III and IV did not. The sedimentation diagram for fraction V is somewhat similar to that of fraction II. These differences indicate that protein precipitated by half saturation of the enzymic digest with magnesium sulphate was not of uniform nature. In the case of fraction II it consisted essentially of relatively larger molecules, whereas the molecular size of the protein in the redispersion of fraction V was a little more heterogeneous, and the redispersions of fractions III and IV contain much greater amounts of low molecular weight substances.

(iii) Experiments on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05

Fermex MT was added to gluten dispersion at 37° C., at the rate of 100 mg. per 100 ml. Aliquots taken at 12 and 36 hours were analysed by sedimentation. Another set of experiments was performed under similar conditions except

that the products of hydrolysis were removed continuously and simultaneously by dialysis. Results are presented in Fig. 9, 10 and Table V.

Table V. Effect of Fermex MT at 100 mg./100 ml. on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05, at 37° C. Sedimentation coefficient of original gluten dispersion = 1.97

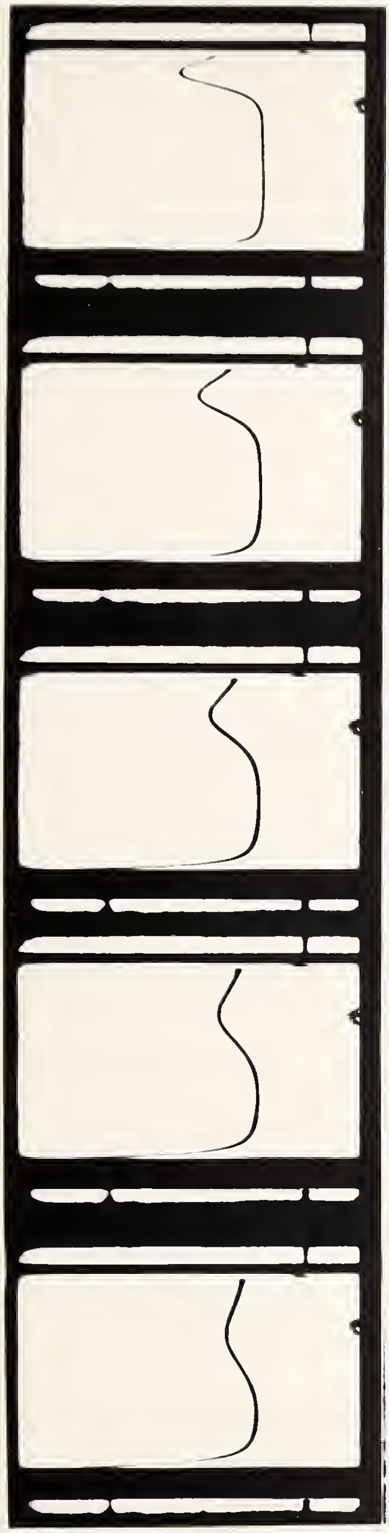
Time of reaction	Sedimentation coefficient	
	after digestion	simultaneously dialysed during digestion
12 hours	1.48	1.52
36 hours	1.23	1.21

The results show that the acid protease of Fermex MT has a somewhat similar action on gluten in aluminum lactate as has the neutral protease. The sedimentation diagrams show only one peak and the sedimentation coefficients are much lower than that of the original dispersion. The sedimentation diagrams, however, also show distinct differences. Those obtained by the action of acid protease on gluten show distinct peaks even at the end of the sedimentation runs, indicating the presence of a substantial proportion of comparatively larger molecules. There was no increase in the sedimentation coefficients as a result of simultaneous

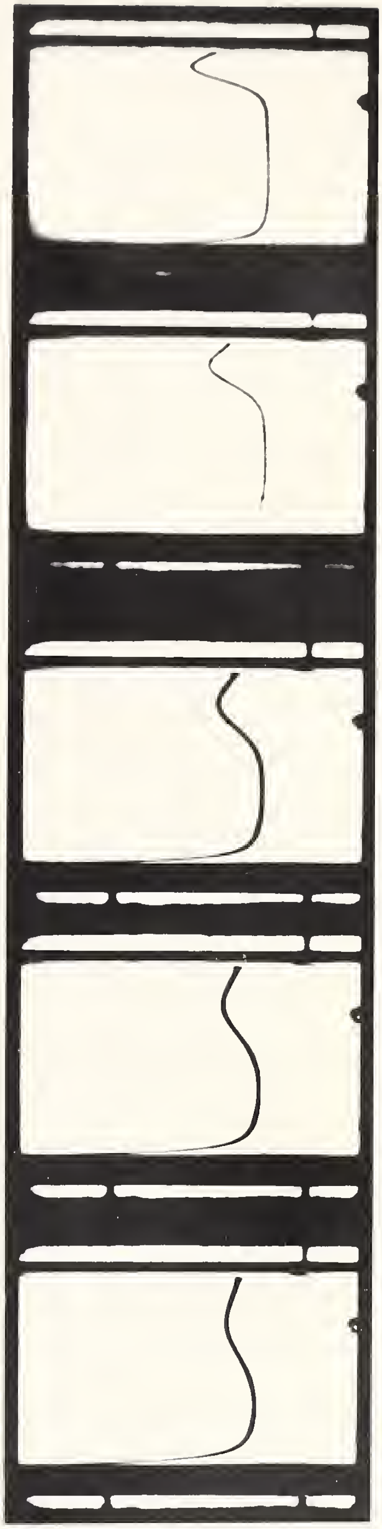
Figure 9. Effect of Fermex MT at 100 mg. per 100 ml., on gluten dispersed in aluminum lactate buffer. First photograph after 57 mins. Others at intervals of 32 mins.

(a) for 12 hours at 37° C.

(b) for 36 hours at 37° C.



(a)

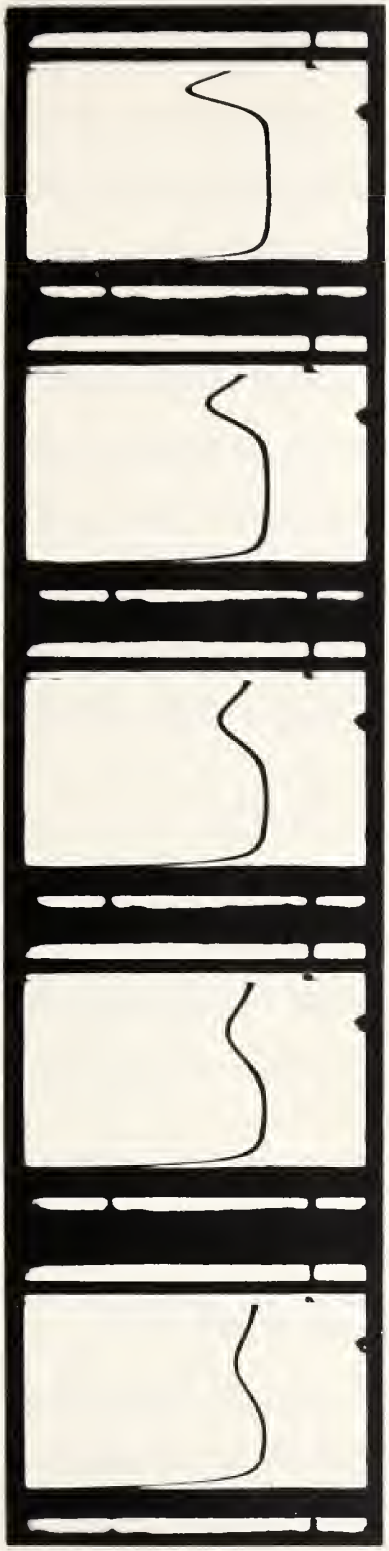


(b)

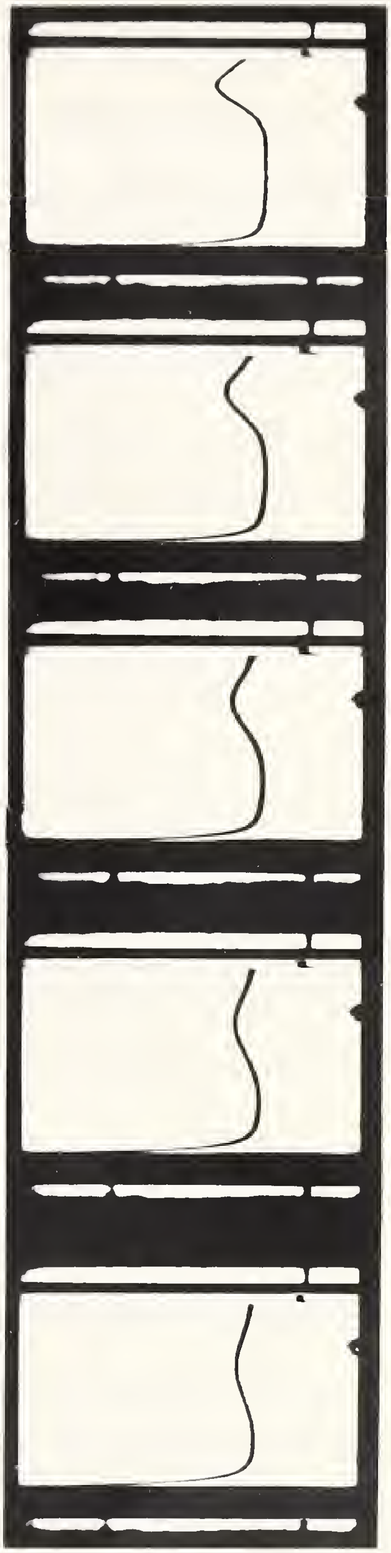
Figure 10. Effect of simultaneous dialysis on Fermex MT digests. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) during 12 hours action at 37° C.

(b) during 36 hours action at 37° C.



(a)



(b)

dialysis though there was an indication of slightly less variation in the molecular size after such treatment. The marked decrease in the sedimentation coefficients of the enzymic digests was associated with the loss of protein during simultaneous dialysis. About 10% of the total protein dialysed out during the first 12 hours, while 26% of the total amount of protein dialysed out during 36 hours. From a consideration of these observations it seems quite clear that a considerable portion of the protein was broken into peptides of higher molecular weight, though material of low molecular weight was also produced and was largely removed during dialysis.

Lack of knowledge of the specificity of the acid protease of Fermex MT does not permit a statement as to the nature of the hydrolysis of gluten by this enzyme, but from the results it appears to be quite different from that obtained at the higher pH in sodium salicylate dispersions.

D. Effects of papain on gluten dispersed in 8% sodium salicylate containing sodium bisulfite

Since the optimum pH of the neutral protease of Fermex MT lies in the pH range in which papain acts, it was decided to investigate the action of this enzyme on wheat gluten. Since papain was shown to react on gluten dispersed in sodium salicylate containing sodium bisulfite, experiments

were conducted with this enzyme in this medium. Because the commercial enzyme preparation was crude and its nitrogen content was rather low (8.6%), an experiment was conducted with 10 mg. papain per 100 ml. dispersion, for 12 hours at 37° C. The sedimentation diagram of the enzymic digest showed that a considerable protein breakdown had taken place and that the sedimentation coefficient could not be calculated. Similar results were obtained with 5 mg. papain per 100 ml. dispersion. When the quantity of papain was reduced to 2 mg. per 100 ml., sedimentation diagrams obtained were such that satisfactory measurements for the calculation of sedimentation coefficients could be carried out. This concentration of enzyme was therefore used in subsequent experiments.

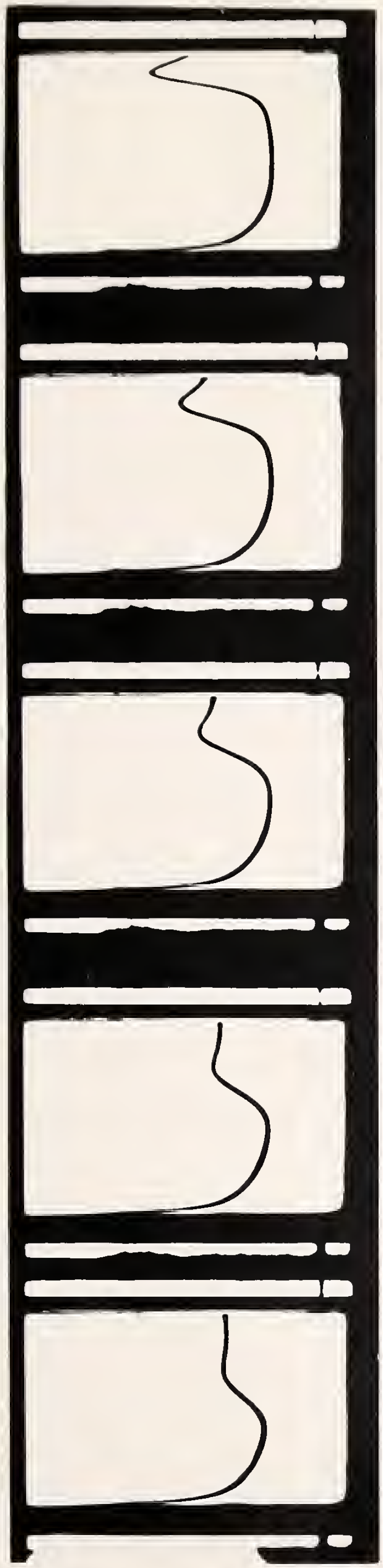
Aliquots of enzymic digests were removed at intervals of 4, 12, and 36 hours for sedimentation analysis. Runs were also carried out on protein precipitated by half saturation of the enzymic digests with magnesium sulphate and redispersed in 8% sodium salicylate. Results obtained are presented in Fig. 11, 12, and Table VI.

Figure 11. Effect of papain at 2 mg. per 100 ml., on gluten dispersed in sodium salicylate containing sodium bisulfite. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) for 4 hours at 37° C.

(b) for 12 hours at 37° C.

(c) for 36 hours at 37° C.



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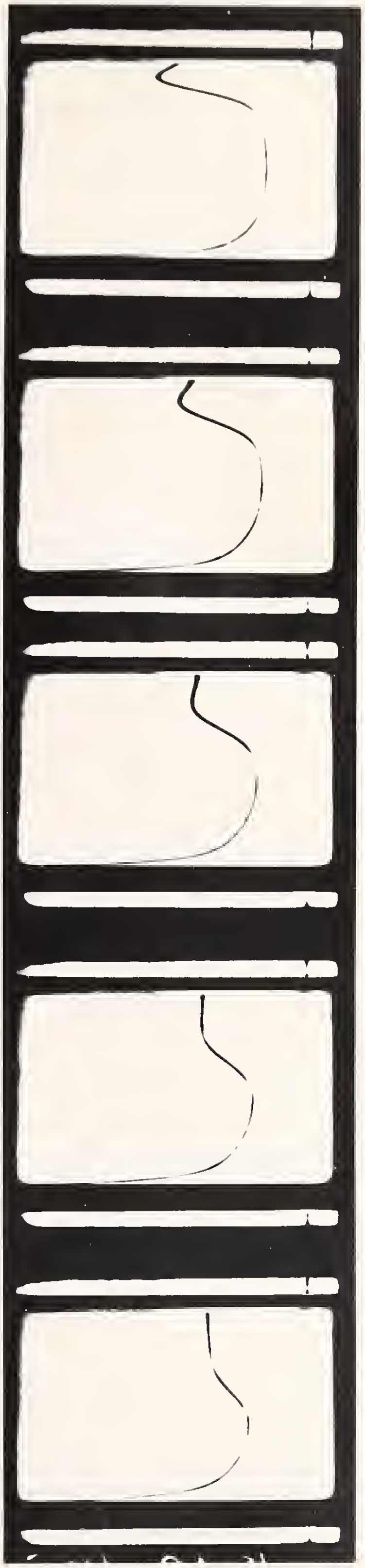

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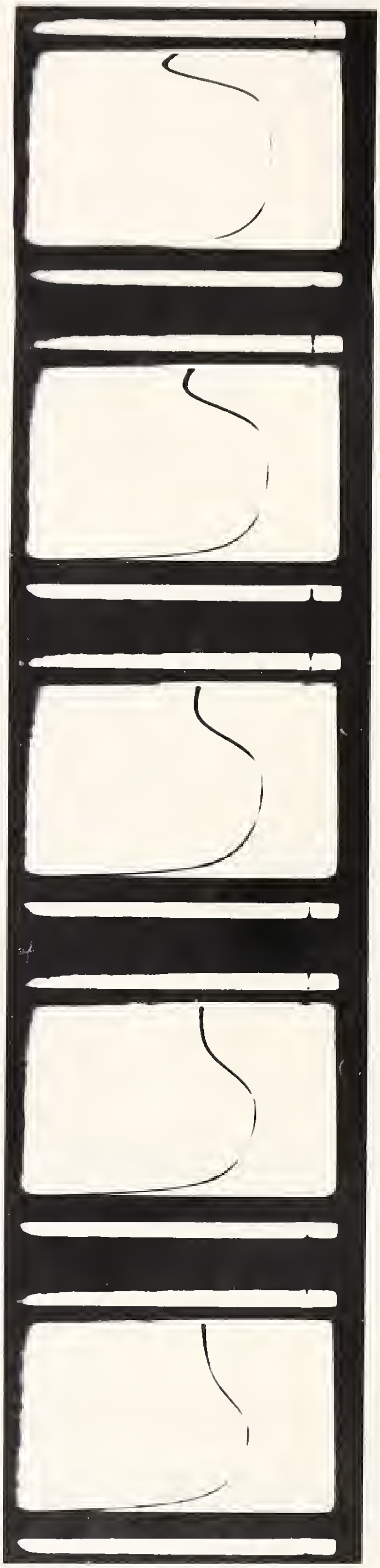
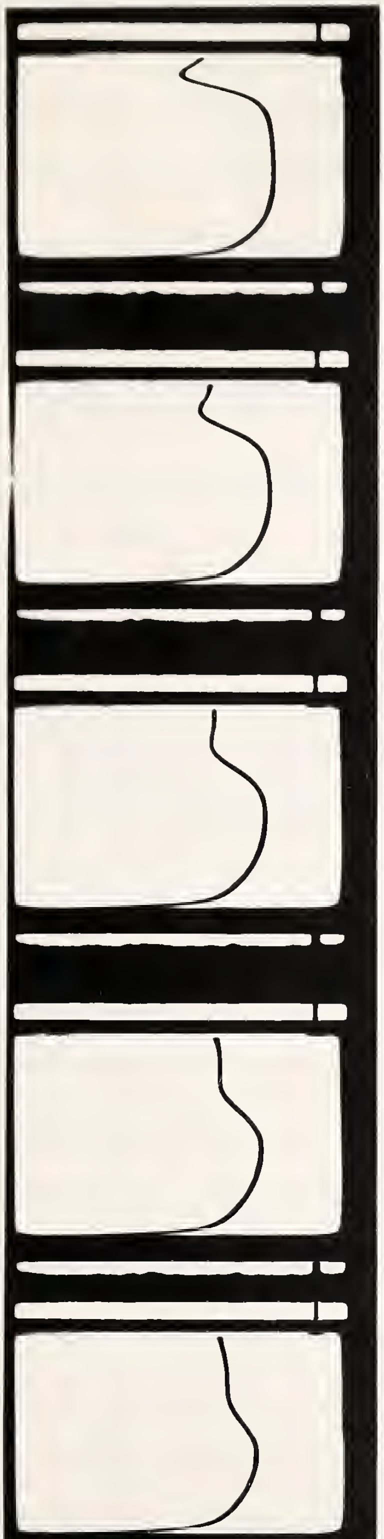


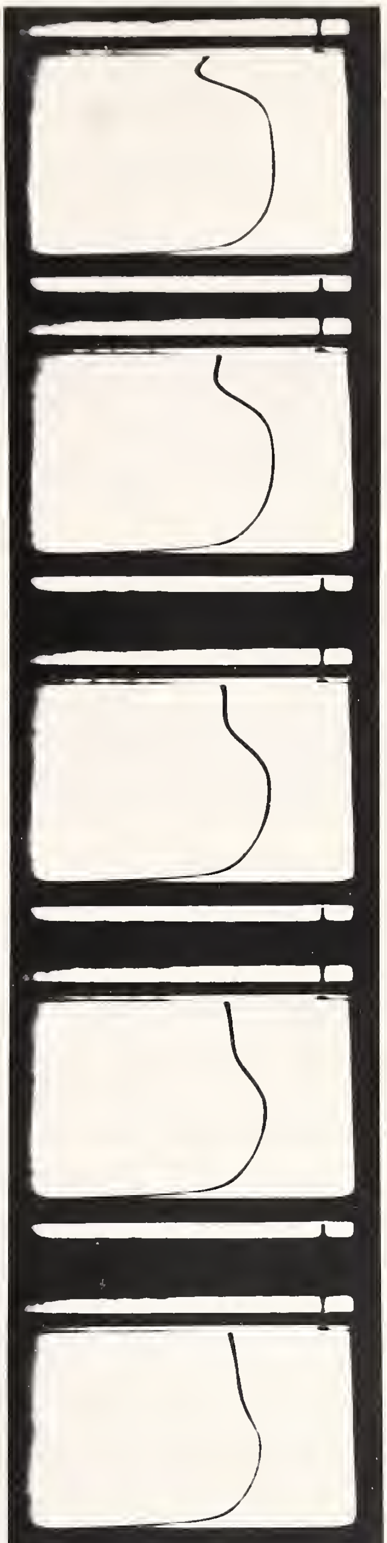
(c)

Figure 12. Effects of precipitation and redispersion on papain digests. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) after 4 hours at 37° C.
(b) after 12 hours at 37° C.
(c) after 36 hours at 37° C.



(a)



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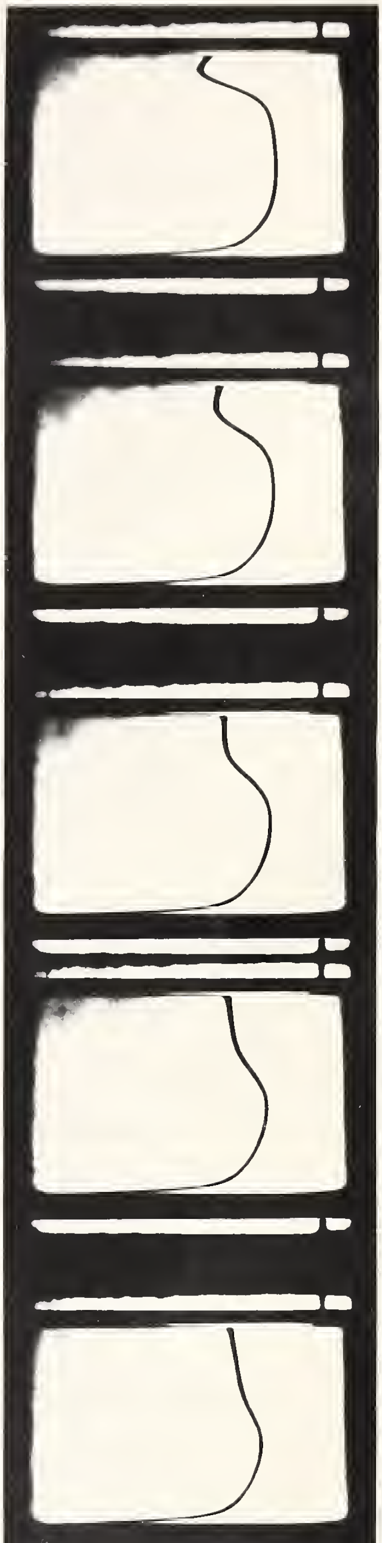


(c)

Table VI. Effect of papain at 2 mg./100 ml. on gluten dispersed in 8% sodium salicylate containing 50 mg. sodium bisulfite per 100 ml., at 37° C. Sedimentation coefficient of original gluten dispersion = 2.11

Time of reaction	Sedimentation coefficient after digestion	Sedimentation coefficient redispersed after digestion	Protein precipitated*
4 hours	1.72	1.73	72.7
12 hours	1.23	1.67	65.6
36 hours	0.96	1.25	47.8

*Amount of protein precipitated by half saturation of enzymic digests with magnesium sulphate, percent of total proteins

The sedimentation coefficient of the enzymic digests decreased as the reaction time increased, indicating a progressive decrease in the mean molecular size. The higher sedimentation coefficients of the redispersed protein obtained after 12 and 36 hours digestion indicates the removal of a considerable proportion of the low molecular weight material. However, there was no increase in the sedimentation coefficient of the redispersed protein after 4 hours reaction, and this suggests that, in the initial stages of the experiment, papain releases largely substances

of low molecular weight. The sedimentation diagrams of the enzymic digests have the characteristic 'sickle' shape. Those of the redispersed protein initially showed well-defined peaks, which flattened as the run progressed, indicating the presence of a considerable proportion of small but variable-sized molecules. The amount of protein precipitated by half saturation of the enzymic digest with magnesium sulphate decreased progressively, showing that the amount of protein broken down to small molecules increases as the time of reaction increased.

In an effort to get a better idea of the pattern of hydrolysis, the rate of reaction was reduced by carrying it out at 25° C. The products of hydrolysis were removed by simultaneous dialysis. Results of the experiments conducted under these conditions are presented in Fig. 13, 14, and Table VII.

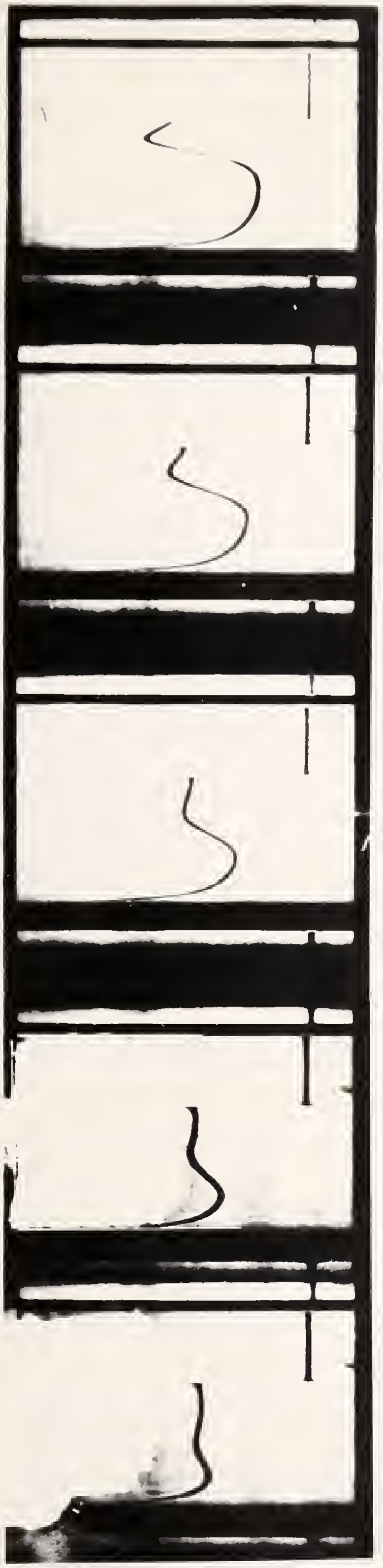
Table VII. Effect of papain at 2 mg./100 ml. on gluten dispersed in 8% sodium salicylate containing 50 mg. sodium bisulfite/100 ml., at 25° C. Sedimentation coefficient of original gluten dispersion = 2.09

Time of reaction	Sedimentation coefficient	
	after digestion	simultaneously dialysed during digestion
12 hours	1.83	1.91
36 hours	1.52	1.90

Figure 13. Effect of papain at 2 mg. per 100 ml., on gluten dispersed in sodium salicylate containing sodium bisulfite. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) for 12 hours at 25° C.

(b) for 36 hours at 25° C.



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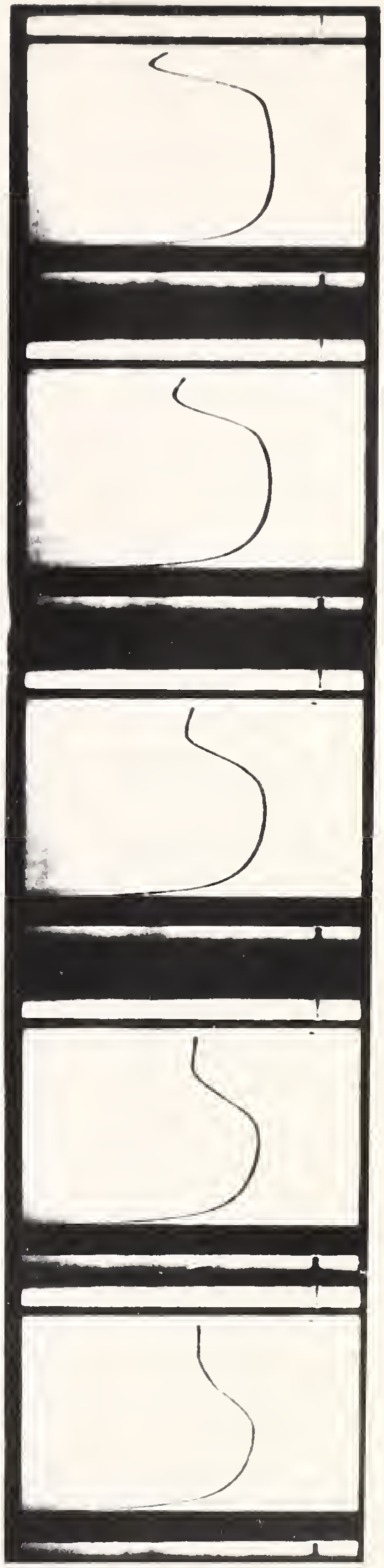
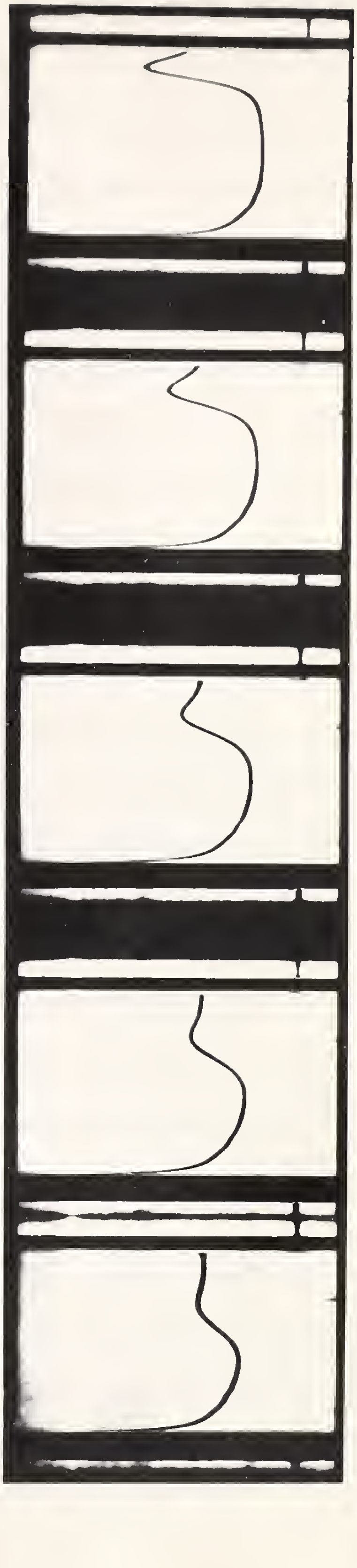


(b)

Figure 14. Effect of simultaneous dialysis on papain digests. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

(a) during 12 hours action at 25° C.

(b) during 36 hours action at 25° C.



The sedimentation coefficients of the enzymic digests are considerably higher than those obtained on the enzymic digests of experiments conducted at 37° C., indicating that the mean molecular size is much higher. The sedimentation coefficient of the simultaneously dialysed digests was also higher than obtained in the earlier experiment, showing that the extent to which gluten molecules were hydrolysed was relatively less. The sedimentation diagrams of the enzymic digests indicate less variation in the molecular size, and those of the simultaneously dialysed digests show well-defined peaks even at the end of the run. Thus, the shape of the diagrams after 36 hours at 25° C. indicates that the hydrolysed portions were removed more completely than were those produced in 4 hours at 37° C. It, therefore, seems possible that the reaction at 25° C. may be somewhat different in nature than that at 37° C.

As in the case of dialysis experiments with the neutral protease of Fermex MT, a bulky precipitate was observed in the digests obtained in these experiments. It is discussed later.

A comparison of the action of papain with that of the neutral protease of Fermex MT does not reveal marked differences. From the amount of protein hydrolysed by the enzymes during the same period, it appears that the action

of papain is a little bit slower, but the concentration of the enzymes may be quite different. Neither the sedimentation coefficients nor the sedimentation diagrams show any definite differences, except perhaps in the initial stages of the experiments. The sedimentation coefficients of the enzymic digest and the redispersed protein after 4 hours' action of papain were identical, showing that the mean molecular size of the principal components in both cases was the same. The sedimentation coefficient of the enzymic digest obtained after 4 hours' action of the neutral protease of Fermex MT was considerably lower than that of the redispersed protein and it was also lower than that obtained on the enzymic digest using papain. This suggests that the enzymic activity of the neutral protease of Fermex MT, during the first four hours, is greater than that of papain.

E. Effects of pepsin on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05

As the optimum pH of the acid protease of Fermex MT lies in the range in which pepsin acts, it was decided to investigate the action of this enzyme on gluten.

Pepsin was added to gluten dispersion at 25° C. at the rate of 20 mg. per 100 ml. Aliquots of the enzymic digests were removed at intervals of 12, 36, and 60 hours for sedimentation analyses. Another set of experiments was

carried out under similar conditions except that the mixtures were contained in cellophane bags and the smaller products of hydrolysis were removed simultaneously and continuously by dialysis. The results of these experiments are presented in Figs. 15, and 16, and in Table VIII.

Table VIII. Effect of pepsin at 20 mg./100 ml. on gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05, at 25° C. Sedimentation coefficient of gluten dispersion = 1.97

Time of reaction	Sedimentation coefficient	
	after digestion	simultaneously dialysed during digestion
12 hours	1.09	1.20
36 hours	0.83	0.89
60 hours	0.71	0.70

The marked reduction in the sedimentation coefficient of the enzyme-treated dispersions indicates an extensive hydrolysis of gluten, which is also shown by the amount of protein which was dialysed out during the course of enzymic digestion. During the first 12 hours of the reaction 7.3% of the total protein had dialysed out. The value increased to 24.4% when the reaction time was 36 hours and at the

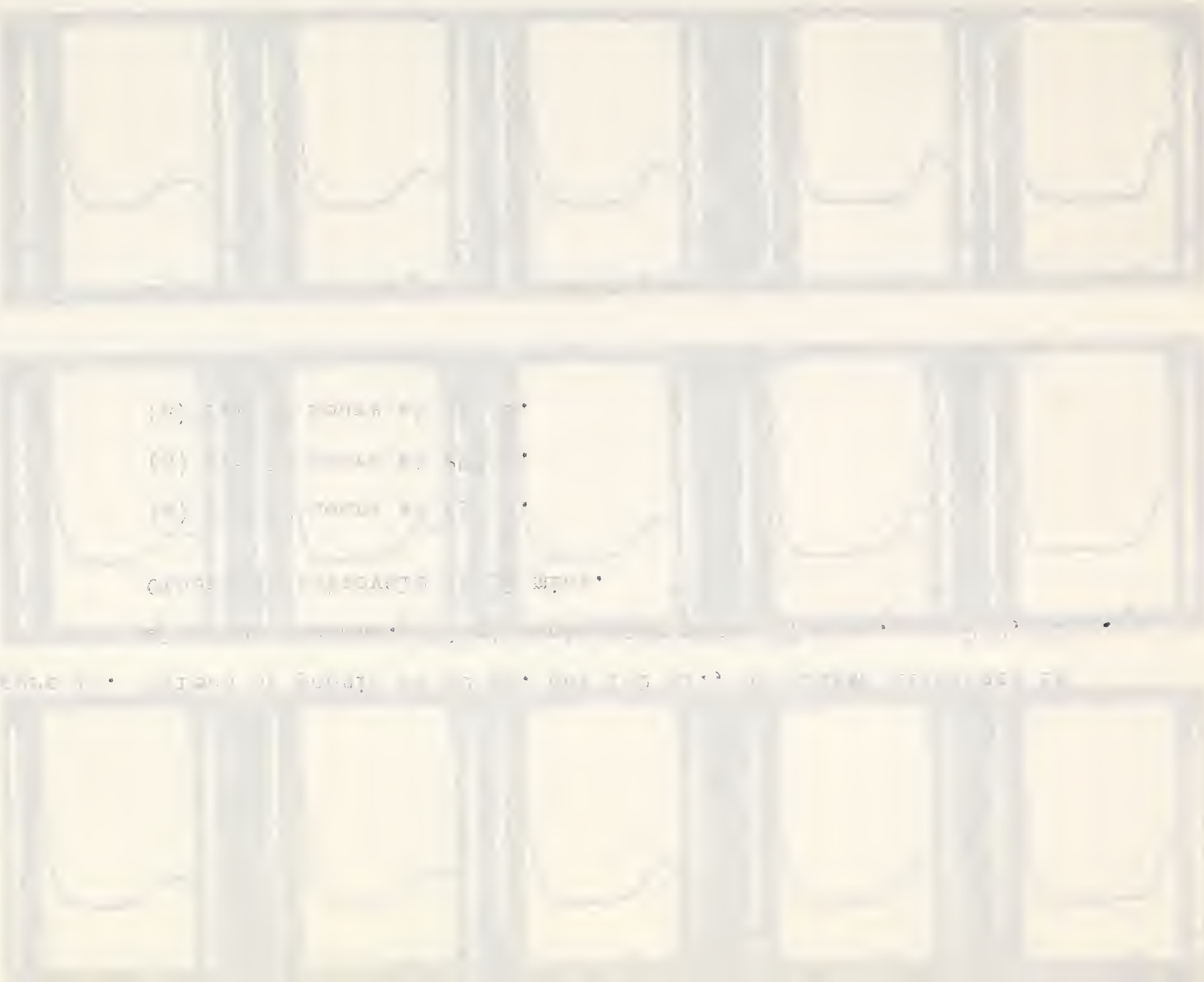


FIGURE [illegible]. [illegible]

(a) [illegible] formed by [illegible].

(b) [illegible] formed by [illegible].

(c) [illegible] formed by [illegible].

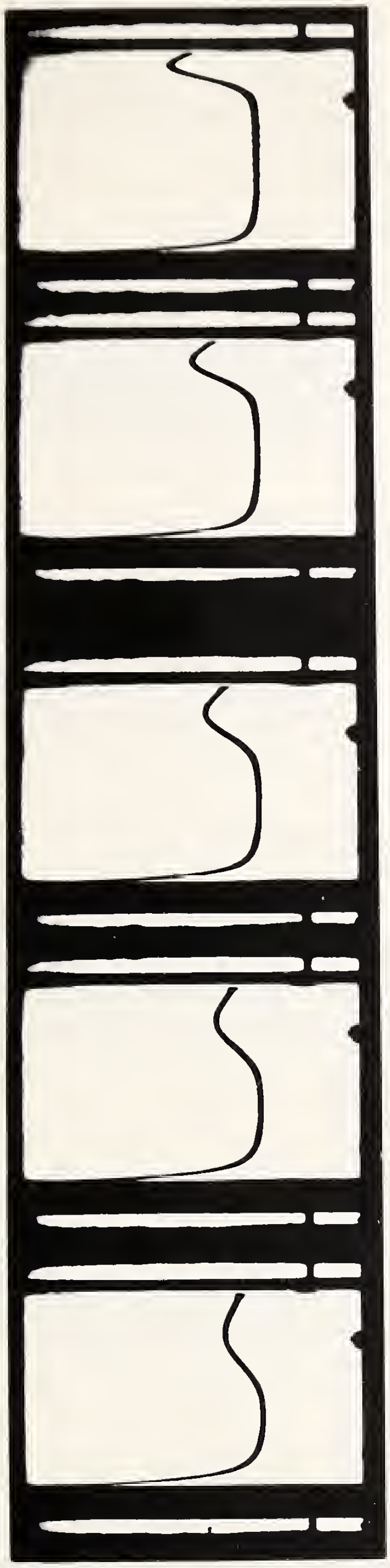
Curves [illegible] represents [illegible].

Figure 15. Effect of pepsin at 20 mg. per 100 ml., on gluten dispersed in aluminum lactate. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

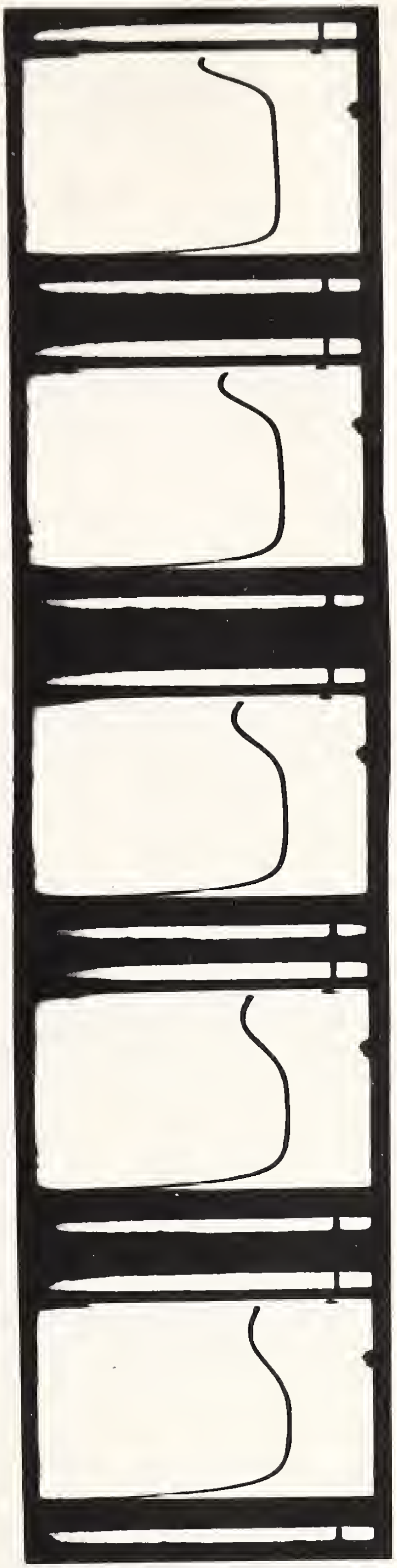
(a) for 12 hours at 25° C.

(b) for 36 hours at 25° C.

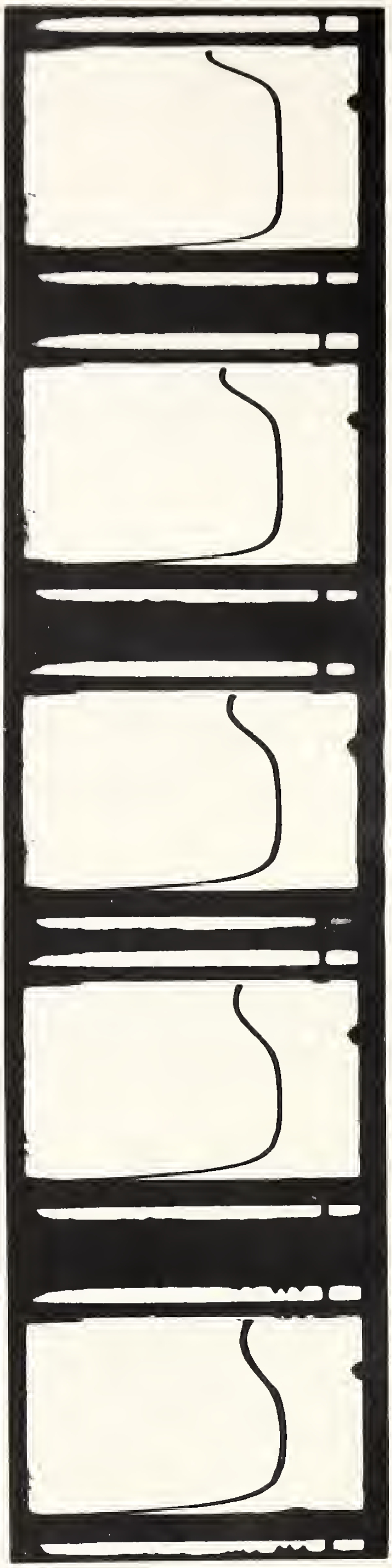
(c) for 60 hours at 25° C.



(a)



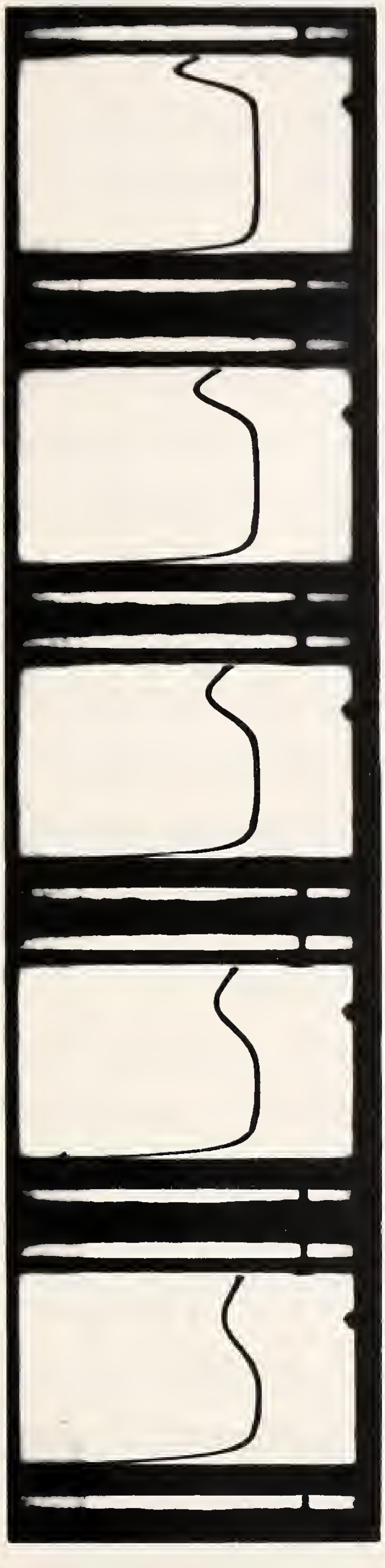
(b)



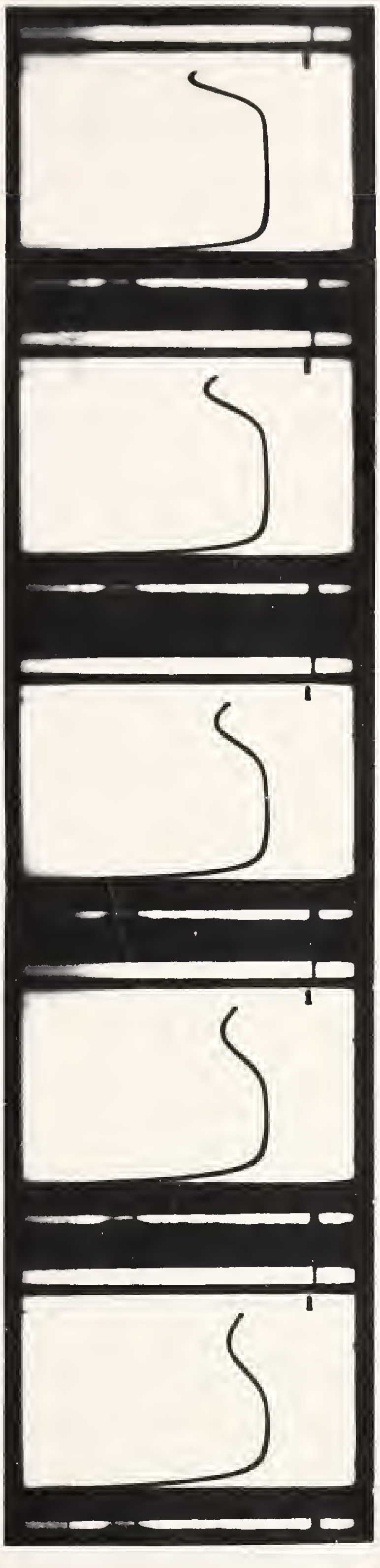
(c)

Figure 16. Effect of simultaneous dialysis on pepsin digests. First photograph after 57 mins., at 250,000 g. Others at intervals of 32 mins.

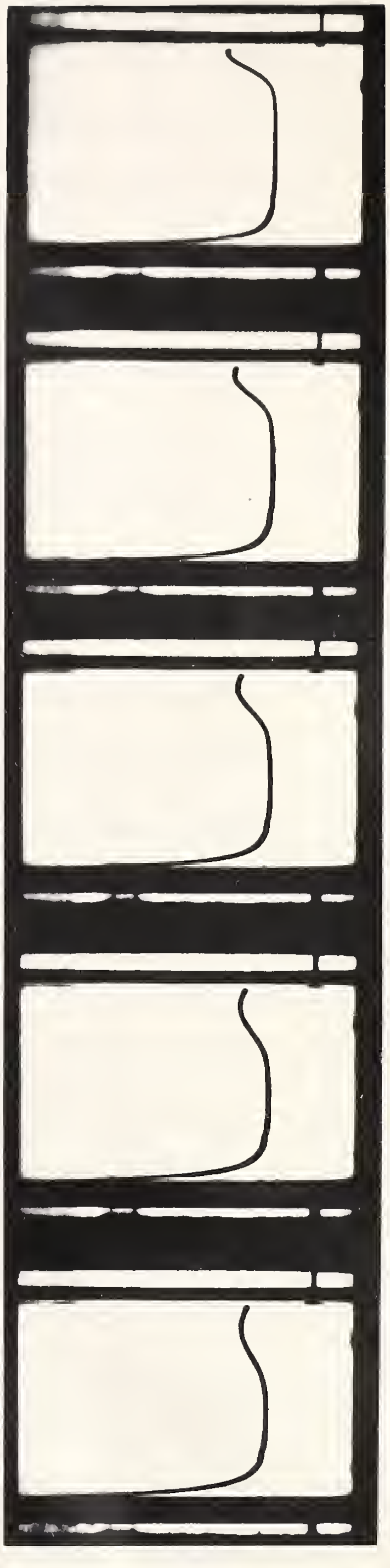
(a) during 12 hours action at 25° C.
(b) during 36 hours action at 25° C.
(c) during 60 hours action at 25° C.



(a)



(b)



(c)

end of 60 hours 55.5% protein had dialysed out. The sedimentation diagrams of the enzymic digests as well as of the simultaneously dialysed digests show that there is less variation in the molecular size than was obtained with the acid protease of Fermex MT, though both sets of the diagrams obtained with pepsin indicate progressive decrease in the molecular size. A few experiments with pepsin were carried out at 37° C. and the results indicate that the rate of hydrolysis is much greater than that obtained with other enzymes. The sedimentation coefficient of the simultaneously dialysed enzymic digest after 12 hours reaction was 1.03 while after 36 hours it was only 0.77. The sedimentation diagrams are not presented here, but they show well-defined peaks similar to those obtained at 25° C. even though the rate of reaction at 37° C. is greater.

Pepsin is known to hydrolyse peptide bonds involving the aromatic amino acids, tyrosine and phenylalanine. In addition to these it has been found to hydrolyse leucyl-valine links, and peptide bonds involving glutamic acid residues (94, p. 266). In view of the fact that gluten is very rich in glutamic acid, the extensive breakdown of the gluten molecule is as would be expected. The reduction of the sedimentation coefficient to 1.09 within the first 12 hours of the reaction indicates that a large number of the peptide bonds are broken in the early stages of the digestion.

Though a strict comparison of the results obtained in these experiments with those obtained with the acid protease of Fermex MT is not possible, yet a consideration of the results show that the action of pepsin is different from that of the acid protease. The acid protease of Fermex MT does not hydrolyse the gluten molecule to the same extent as does pepsin. The mean molecular size obtained with the former is considerably larger than that with the latter. Even though the amount of protein removed during dialysis experiments with both the enzymes is approximately the same, the shape of the sedimentation diagrams show the presence of a greater proportion of small but non-dialysable molecular weight material in the digests obtained by the action of the acid protease of Fermex MT.

F. Properties of the enzyme resistant 'core'

Extensive investigation of the insoluble material obtained during dialysis experiments with the neutral protease of Fermex MT, papain and pepsin, could not be carried on because of its insolubility in sodium salicylate with and without bisulfite, in aluminum lactate buffer and in dilute acids. The enzyme resistant 'core' was, however, soluble in dilute alkali. In order to get some indication as to the nature of the material it was hydrolysed with 20% hydrochloric acid for 24 hours and its total nitrogen, and

amide-nitrogen content were determined. It was found that the insoluble material amounted to 10 - 15% of the total nitrogen in the dispersion, and that it was much lower in its amide-nitrogen content than gluten. The amide-nitrogen content of the insoluble material was about 16.0% as compared to about 24.0% of gluten.

A better idea of the nature of the insoluble material may be provided by amino acid analysis. Such analyses were available only after all experiments were finished and on a limited scale. The results, though significant, must be treated as strictly preliminary.

Insoluble material obtained after 60 hours action of papain and 36 hours action of the neutral protease of Fermex MT, both at 25° C., was hydrolysed for 24 hours in 20% glass-distilled hydrochloric acid. The hydrolysates were evaporated to dryness on a rotary evaporater and the residue taken up in 0.1 M citrate buffer, pH 2.2. Amino acids were determined on an automatic amino acid analyser manufactured by Spinco Division of Beckman. The results obtained are presented in Table IX together with the results obtained on crude gluten hydrolysate.

The results show some striking differences. The values for ammonia, which indicates the amide-nitrogen content

Table IX. Amino acid composition of crude gluten and enzyme resistant 'core'

Amino acid	Gm. amino acid/100 gm. protein (N x 5.7)		
	Crude gluten	Papain insoluble	Fermex MT insoluble
Lysine	1.61	4.24	4.62
Histidine	2.01	2.91	3.09
Ammonia	4.33	3.01	2.95
Arginine	3.39	7.03	7.17
Aspartic acid	2.75	7.09	7.38
Threonine	2.46	3.61	3.72
Serine	4.63	5.42	5.38
Glutamic acid	37.06	27.08	24.96
Proline	12.69	8.11	7.97
Glycine	3.35	4.22	5.38
Alanine	2.51	4.64	4.89
Cystine (1/2)	1.28	1.41	1.46
Valine	3.75	5.66	5.47
Methionine	1.50	2.27	2.41
Isoleucine	3.68	4.61	4.69
Leucine	6.81	9.16	8.91
Tyrosine	3.46	4.66	4.68
Phenylalanine	4.98	5.64	5.63
Total	102.25	110.77	110.76

show that the insoluble material contained much lower amounts of amide-nitrogen. The amount of arginine in the insoluble material was found to be over twice as much as in crude gluten. The glutamic acid and proline content of the insoluble material was much lower than in crude gluten hydrolysate. On the other hand, the amount of other amino acids was much higher in the insoluble material than in crude gluten hydrolysate. The increase is very pronounced for lysine, aspartic acid, alanine and leucine. Similar results have been reported by Stauffer *et al*. (62) for gluten fraction I, and by Meredith *et al*. (66) for fraction B. McCalla and associates (55, 56) have also shown that the more insoluble gluten fractions are high in arginine content and low in amide-nitrogen content. These results suggest that the insoluble material after enzymic digestion is similar to the more insoluble fractions of gluten. This may seem not to agree with the results obtained with gluten fractions, where the more insoluble fraction was digested to a greater extent by the neutral protease of Fermex MT than were the more soluble ones (P. 55). However, the more insoluble fraction contained about four times as much protein as the insoluble 'core' obtained on proteolytic digestion of gluten. Strusi (49) reported the amino acid analysis of the acid hydrolysates of the residue of tryptic digestion of gluten from bread and durum wheats. His results are qualitative and do not provide any additional information.

Detailed analysis of gluten fractions and the insoluble material is required for a definite statement regarding the nature of the enzyme resistant core. The significance of this material is considered in the General Discussion.

GENERAL DISCUSSION

Both proteolytic enzymes and reducing agents cause changes in the physical characteristics of dough when added to it (19, 28), but the action of reducing agents can be reversed by subsequent oxidation whereas that of the proteolytic enzymes cannot be reversed (32). The action of proteolytic enzymes differs from that of reducing agents also in being progressive, as the amount of gluten recovered from enzyme-treated doughs decreases with time (36, 37). Results supporting these observations have been obtained in the present investigation. In preliminary experiments it was observed that the incorporation of sodium bisulfite in sodium salicylate solution decreases the time of dispersion roughly from 6 hours to 2 hours. When gluten dispersions obtained with and without bisulfite were sedimented, no differences in the sedimentation characteristics were observed (Fig. I). This indicates that the action of sodium bisulfite is primarily a physical disintegration of gluten. Papain appears to have no action on gluten dispersed in sodium salicylate in the absence of bisulfite (Fig. 2b). However, when the sedimentation diagrams of Fig. 3 are considered, it is clear that the action of papain is entirely different from that of sodium bisulfite. The sedimentation coefficient of papain-treated dispersion is much lower than that of gluten

dispersed with or without bisulfite, and shows a progressive decrease with time (Table VI). This is also borne out by the progressive decrease in the amount of protein precipitated from the enzymic digest by 0.5 saturation with magensium sulphate (Table VI), while just as much gluten is precipitated from the bisulfite-containing dispersions as from sodium salicylate alone.

Having established the differences in the action of papain and reducing agents, an attempt should be made to explain the mechanism of action of reducing agents.

Reducing agents rupture the disulfide bonds of the protein molecule. Gluten is thought to contain some disulfide bonds. It has been suggested (95, 96) that the reducing agents break some of these bonds in the gluten molecule. If the reducing agents rupture the interchain disulfide bonds, the size of the molecule will be reduced (e.g. in insulin), resulting in a lowering of sedimentation coefficient, a result which has not been observed in this investigation. There is a possibility that the gluten molecule has intra-chain disulfide bonds and that these are broken. If this happens, the molecular size will not change even though the shape might be somewhat altered. Hence, the sedimentation characteristics of gluten treated with the reducing agents will be similar to that of untreated gluten, although the

rate of dispersion is decidedly increased. This argument seems compatible with the results obtained in the present investigations.

Olcott *et* *al*. (31) claim that the proteolytic enzyme of gluten is reversibly inactivated by sodium salicylate. Their results show that the enzyme is one which acts in acidic medium. Both papain and the neutral protease of Fermex MT act on gluten in 8% sodium salicylate containing bisulfite (Fig. 3), indicating that proteolytic enzymes can act in this medium. Pepsin, on the other hand did not act on gluten in this solvent but did act on gluten in an acidic medium - 0.02 M aluminum lactate buffer, pH 3.05 (Fig. 4). This observation indicates that sodium salicylate itself probably has nothing to do with the inactivity of the enzyme, but that the proteinase present in gluten acts only in an acidic medium.

It has been claimed (8, 22, 23) that glutenin is not affected by proteolytic enzymes. This is based on the observation that the amount of glutenin obtained after the action of proteolytic enzymes did not change. Extensive investigation of this aspect has not been carried out, but the results of the proteolytic digestion of gluten fractions do not support the claim. Results presented in Table IV show that the comparatively insoluble fraction of gluten,

fraction II, is digested to a greater extent than the other three fractions. Only 67% of the total protein was precipitated on half saturation of the enzymic digest of fraction II with magnesium sulphate, while about 80% of the protein was precipitated under similar conditions from enzymic digests of the other three fractions. The observations of Sharp and Elmer (8), and Blagoveschensky and Yurgenson (22, 23) may be explained by the results of simultaneous dialysis experiments. It was found that a portion of the protein precipitated during dialysis of gluten dispersions containing enzymes. This precipitate was insoluble in sodium salicylate, aluminum lactate, and dilute acids, but dissolved readily in dilute alkali. This precipitate contained 10 - 15% of the total nitrogen of the dispersion. The amide-nitrogen content of the precipitate was also low. It seems that this enzyme resistant 'core' insoluble in many conventional solvents, low in amide-nitrogen, may be the substance which led Sharp and Elmer, and Blagoveschensky and Yurgenson to postulate that glutenin is resistant to enzymes. Insoluble material was also found by Strusi (49) on tryptic hydrolysis of gluten from durum and bread wheats. In any case, it seems quite certain that the part of gluten commonly termed "glutenin" is not immune to enzyme attack under the conditions of the present investigations.

The results presented in the thesis have provided preliminary information regarding the proteolysis of wheat gluten. A better understanding of the problem would, however, require extensive investigations on a number of aspects. The separation and characterization of the products of hydrolysis has received some attention (49), but a detailed physico-chemical characterization of the products of hydrolysis is required. The kinetics of enzyme reactions also merit investigation.

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APPENDIX

Determination of partial specific volume of gluten dispersed in 0.02 M aluminum lactate buffer, pH 3.05

In order that the observed sedimentation coefficient values be comparable they have to be reduced to a standard, usually $S_{20,w}$. This involves correction for the temperature, for the viscosity of the solvent as compared to water, and the partial specific volume. Because most of the sedimentation velocity runs were made at 20° C., the correction for temperature was not necessary. Viscosity of the solvents was determined at 20° C. and was found to be respectively 1.035 and 1.146 for aluminum lactate and sodium salicylate containing sodium bisulfite. Partial specific volume for gluten dispersed in 8% sodium salicylate was determined by McCalla and Gralén*, but the value for gluten in aluminum lactate buffer is not recorded, hence it was determined.

Determination of partial specific volume involves the determination of the densities of the solvent and of the solution, preferably at different concentrations.

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*McCalla, A.G., and Gralén, N. Ultracentrifuge and diffusion studies of gluten. Can. J. Res. 20:130-159. 1942.

These values are substituted in either one of the following formulae* to obtain an apparent partial specific volume value for the particular concentration:

$$V_{app.} = \frac{100/d - (100 - n)/d_o}{n} \quad . \; . \; . \; . \; . \; (1)$$

or

$$V_{app.} = \frac{1}{d_o} - \frac{1}{x}\,\frac{(d - d_o)}{d_o} \quad . \; . \; . \; . \; . \; (2)$$

where d and d_o are the densities of the solution and solvent respectively, n is the percent concentration of the solute by weight, and x is the concentration of protein in gm./ml.

Density determinations were carried out at 25° C. in a 50 ml. pyconometer. The concentration of the solute was determined by the nitrogen content of the solution (micro-Kjeldahl) x 5.7. The values so obtained were substituted in formula (2) for calculation of the apparent partial specific volume for each concentration. The values obtained are given below:

Percent protein concentration	$V_{app.}$
0.687	0.710
1.015	0.703
1.55	0.712
1.858	0.711
3.172	0.692

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*Schachman, H.K. Ultracentrifugation, diffusion and viscometry. In Methods in Enzymology. V. IV. Academic Press. 1957. pp. 32-103.

The average value obtained from these figures is 0.706 ml. per gm. and was used in the correction of observed sedimentation coefficient values to $S_{20,w}$.

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